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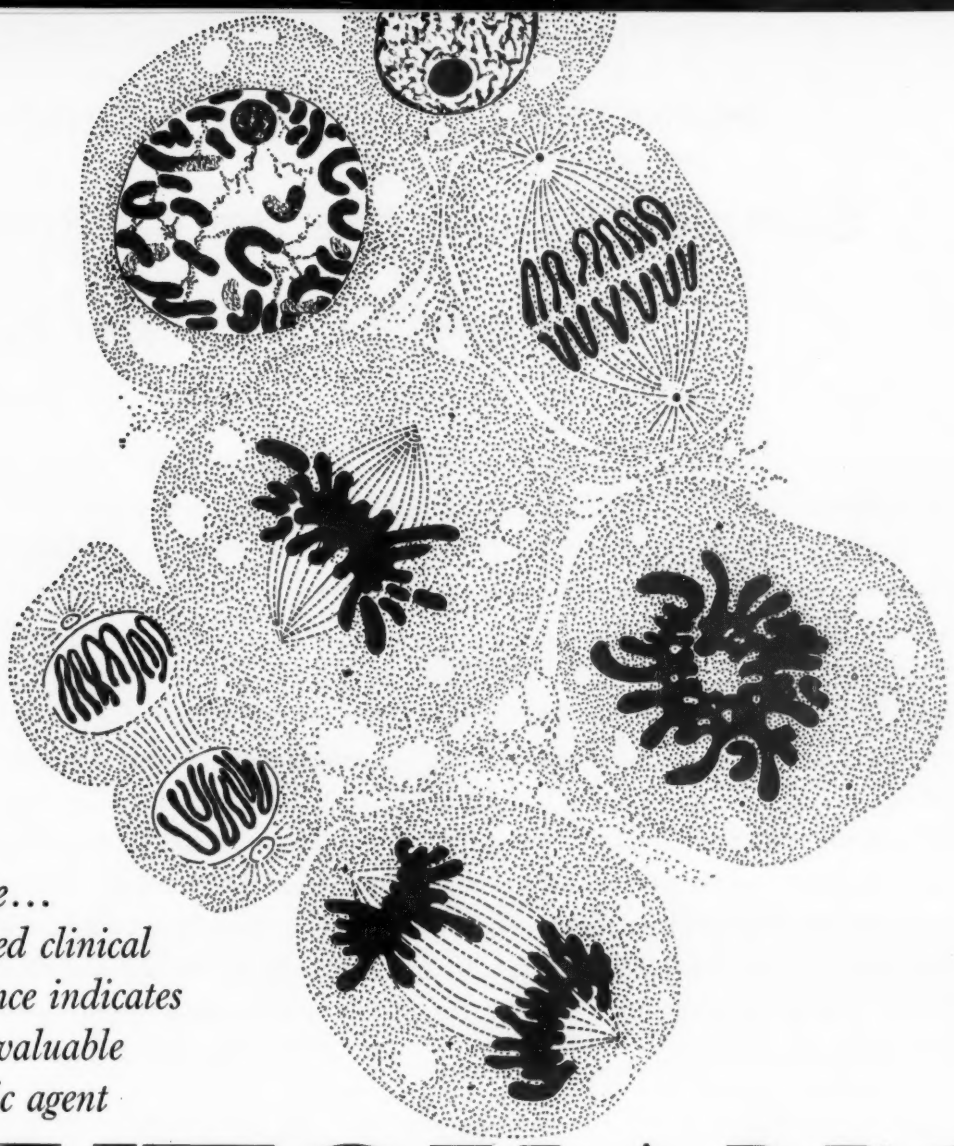

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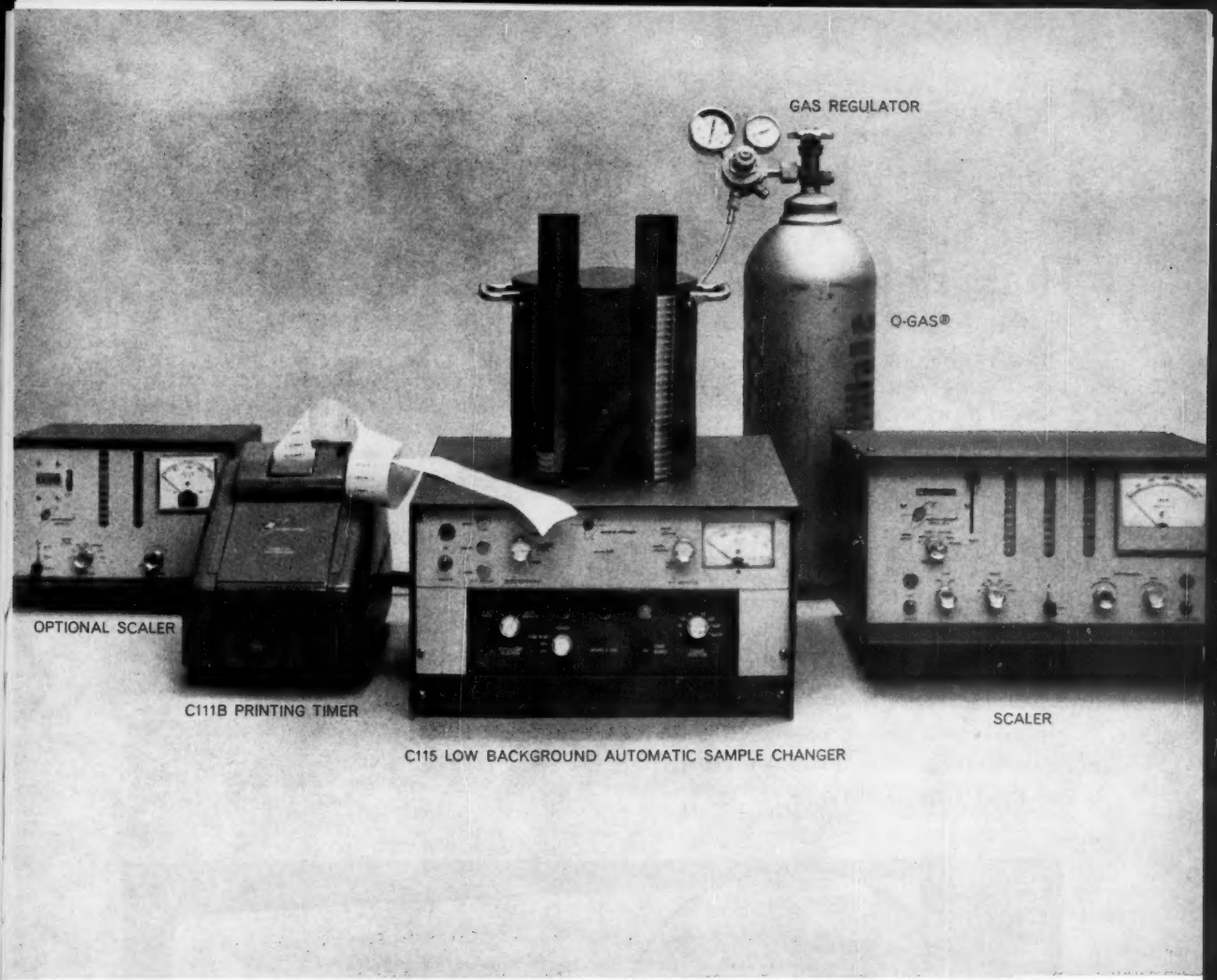

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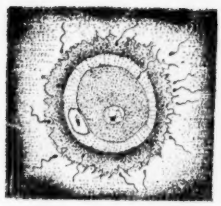

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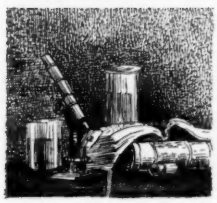

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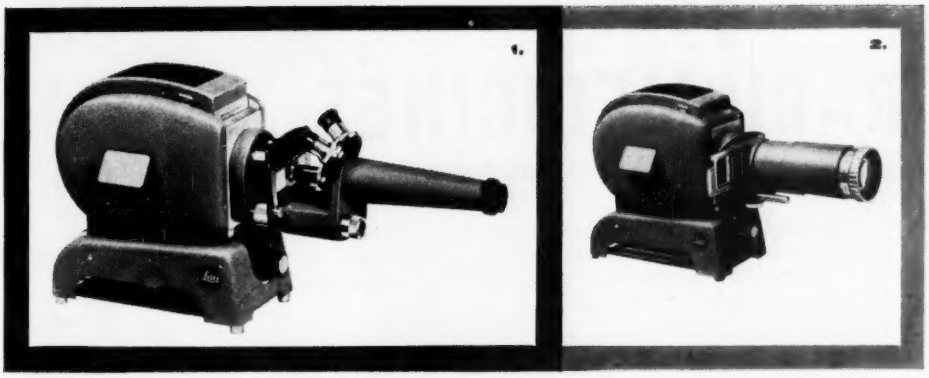

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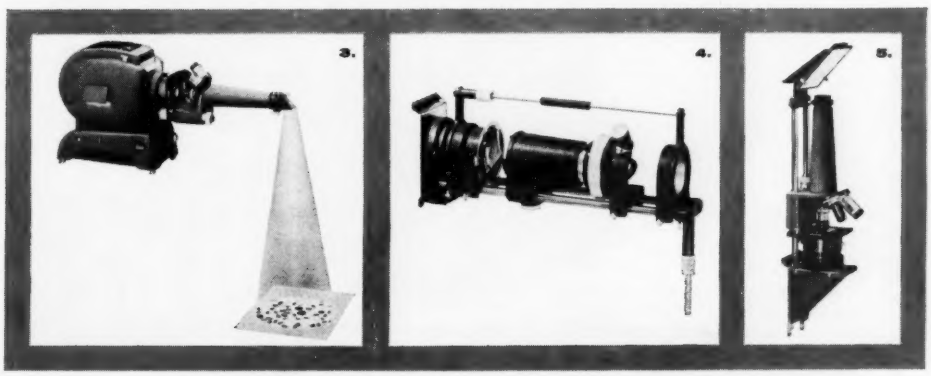

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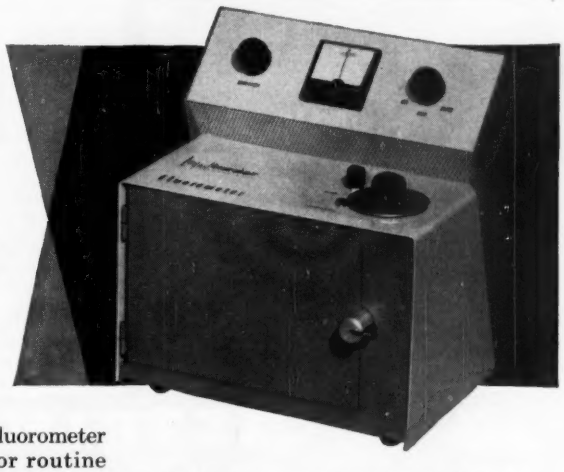

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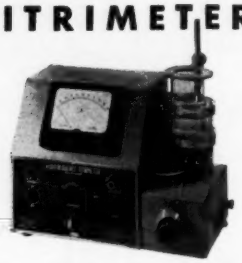

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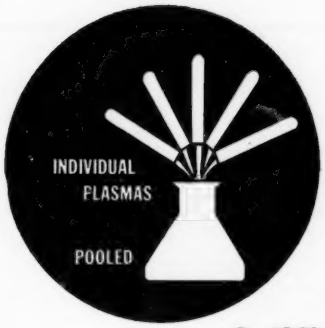

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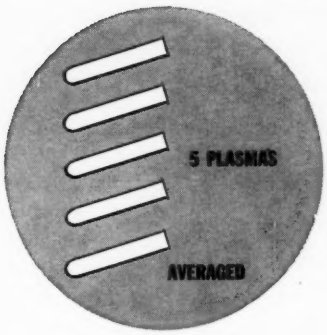

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THE CHARACTERISTICS OF THE PERIPHERAL TRANSPORT OF C^{14}–LABELED PALMITIC ACID *

By S. J. FRIEDBERG,† R. F. KLEIN, D. L. TROUT, M. D. BOGDONOFF AND E. H. ESTES, JR.

(From the Central Reference Laboratory and Radioisotopes Unit, Veterans Administration Hospital, and the Department of Medicine, Duke University Medical Center, Durham, N. C.)

(Submitted for publication April 21, 1960; accepted June 9, 1960)

Previous studies of the uptake and release of free fatty acid (FFA) of various tissues (1, 2) have indicated that during the basal state there is usually a net release of FFA from the leg, but a more variable response in the forearm. This variability of forearm arteriovenous FFA differences has raised the issue of whether or not arteriovenous differences of total FFA alone provide an accurate index to the dynamics of FFA transport across peripheral areas. The studies reported here indicate that the characteristics of C^{14}-labeled palmitate transport may be disparate with arteriovenous differences of total FFA, and that evidence of tissue uptake of FFA may be apparent in the presence of negative arteriovenous differences of total FFA.

METHODS AND MATERIALS

Studies were carried out in male hospital patients who were above age 35 and who had been fasted for 10 to 15 hours. All but one subject were free of serious disease and none was diabetic.

Sixteen subjects were studied by infusing into an arm vein 0.004 to 0.010 mc of albumin-bound palmitic acid-1-C^{14} by means of a Bowman constant infusion pump.[1] Individual dosage was determined according to age. One seriously ill aphasic patient with a progressive degenerative central nervous system disorder received 0.030 mc over a period of 3 hours. The albumin-bound palmitic acid-1-C^{14} was prepared as previously described (3) and mixed with an appropriate amount of physiological saline solution so that the desired amount of label could be administered at the rate of 2.1 ml of solution per minute for a varying length of time (20 minutes to 3 hours). Simultaneous 6 ml arterial and venous blood samples were collected in syringes at suitable intervals from indwelling needles either from a brachial artery and a convenient antecubital vein or from a femoral artery and vein in the same limb. No samples were drawn from the arm being infused and no attempt was made to sample from veins which were thought to drain specifically deep forearm tissue or muscle. In 6 subjects, sampling was continued after the infusion was stopped. The use of a tourniquet to collect venous blood was generally avoided; however, when the use of a tourniquet became necessary no inconsistencies were noted in the results.

FFA concentration was determined by the method of Dole (4). Palmitic acid-1-C^{14} was extracted, separated from other lipids, and measured as previously reported in a windowless gas-flow counter (3). Later a Packard tri-carb liquid scintillation spectrometer was used. Samples were prepared for this detector essentially in the same manner with the following exceptions: the heptane extracts of fatty acids, together with 12.5 mg of palmitic acid carrier, were put into scintillation vials. The solvents were evaporated by blowing a stream of cool air from a hair dryer across the mouth of the vials. Ten ml of toluene-phosphor solution was then added and the samples counted to a maximum error of 3 per cent. Addition of an internal standard revealed no significant quenching.

RESULTS

During constant infusion the arterial level of label rose rapidly at first and reached a value in about 12 to 15 minutes[2] from which it increased

* This investigation was supported in part by a research grant from the Life Insurance Medical Research Fund, in part by a research grant H-1217 (C6) from the National Heart Institute, Bethesda, Md., and in part by the Regional Center for the Study of Aging, Duke University, Durham, N. C.

† Research Fellow, American Heart Association.

[1] Palmitic acid-1-C^{14}, specific activity, 1 mc per mmole, obtained from Volk Radiochemical Co., Chicago, Ill.

[2] If a substance is injected into a fixed volume "v" at the rate of "a" units per unit time, and a constant fraction "K" of the amount present is removed per unit time "t," the amount of the substance "x" present at any time is equal to $(a/K)\ (1-e^{-Kt})$. Also, if "c" is the concentration of label, and "v" its volume of distribution, $c = (a/Kv)\ (1-e^{-Kt})$ (5).

When $t = \infty$, $e^{-Kt} = o$ and $x = a/K$.

When the concentration of label becomes constant, (at $t = \infty$) the rate of influx equals the rate of efflux. If one selects arbitrary values for t, a, and K, and plots $x = (a/K)\ (1-e^{-Kt})$, x will rise rapidly at first and then

TABLE I

Fractional disappearance rates and plasma flux of free fatty acids (FFA) calculated by the method of constant infusion and data used in these calculations

Subject	Arterial plasma FFA concentration	C^{14} activity per estimated plasma vol. of 3,000 ml	Rate of infusion of palmitic acid-1-C^{14}	Fractional disappearance	Plasma FFA flux
	μmoles/L	*cpm*	*cpm/min*	*rate/min*	*μmoles/min*
J.C.	610	386,000	87,300	0.23	420
J.Q.	660	454,000	121,200	0.28	550
P.B.	450	375,000	115,500	0.31	420
C.H.	335	457,000	106,700	0.24	250
F.D.	830	233,000	74,000	0.32	850
C.A.	540	129,000	56,800	0.43	700
W.C.	720	225,000	49,300	0.22	480
A.W.	230	99,000	45,300	0.45	310
R.C.	712	115,000	35,700	0.31	660
H.P.	854	82,700	27,700	0.33	850

only very slowly (Figures 1 and 2). In all subjects the venous level remained less than the arterial level by an amount which varied considerably from subject to subject, but the amount of the arteriovenous difference did not exceed 50 per cent of the arterial level. Table II lists the mean values of the labeled palmitate and titrated total plasma FFA for arterial and venous blood after equilibrium had been reached. With constant infusion of label there was always a positive arteriovenous difference of palmitic acid-1-C^{14} regardless of whether the total FFA arteriovenous difference was positive (Figure 1A) or negative (1B). In those subjects in whom there was a positive arteriovenous difference of total FFA, the relative magnitude of the arteriovenous difference of label exceeded the total FFA arteriovenous difference; the amount of label extracted always pointed to a greater removal of fatty acid than was indicated by the plasma FFA arteriovenous difference. Examination of Figure 1 shows that palmitic acid-1-C^{14} levels, both arterial and venous, seem to rise very slowly during prolonged administration. Since some of the data suggest that arteriovenous difference might become narrower with time, one subject (L.C.) was infused with tracer for 3 hours to see whether or not the positive arteriovenous difference could be abolished. However, the arteriovenous difference was maintained for the entire time with minor fluctuations. We have no explanation for this gradual very slow rise, which was not a constant observation. The possible roles of recirculating label, of nonremovable label and of changing physiological state might be considered.

When the constant infusion was stopped, the arterial and venous concentrations of label fell rapidly and the positive arteriovenous difference immediately disappeared or, in most instances, became slightly negative (Figure 2). These events are not felt to be the result of recirculation of label; the mean circulation time through the arm as measured by Freis, Schnaper and Lilienfield (7, 8) is greater than 30 seconds. In a system in which arterial level is falling rapidly a delay of this magnitude would make it appear as if a positive arteriovenous difference were abolished.

slowly approach limit a/K asymptotically. The behavior of constantly infused label appears to be analogous. From a practical standpoint, the concentration changes very little or rises slowly enough so that the level of label can be considered to be a/K after about 30 minutes. At an average FFA fractional disappearance rate of 0.3 per minute the level of label can be expected to reach 99 per cent of the limiting value a/K in 15 minutes.

It might be tempting to calculate flux of FFA from constant infusion data and to compare these calculations with values obtained by single injection methods. However to do this one would have to know the volume of distribution of FFA, or v. This quantity is not necessarily identical with the intravascular volume. Furthermore, the average concentration of palmitic acid-1-C^{14}, c, cannot be obtained readily because the concentration of this label in the blood is anything but uniform; arterial level is not representative of the blood as a whole and neither is the mixed venous blood which contains the newly infused label. The best one can achieve is an approximation. These approximations measured from our data do fall within the range of the values obtained by single injection (6) but we do not feel that these are reliable calculations (Table I). Using the same equation, an estimate of the volume of distribution of FFA indicates that this volume is about the same size as the blood volume or slightly larger.

Furthermore, the slow component of the forearm circulation which produces a delay of about 2 minutes might play an important role in apparent recirculation of label. These observations will be discussed elsewhere in greater detail (9).

DISCUSSION

The objective of previous studies of arteriovenous differences of total titratable free fatty acids in the peripheral areas during the basal state has been to delineate the direction and, to some extent, the magnitude of FFA transport in those areas. The arteriovenous difference across the extremities during conditions of relatively unchanging blood flow has been felt to represent an index of tissue utilization of the substance under study (1, 2). Andres, Cader and Zierler used arteriovenous differences of glucose across the forearm to calculate that component of oxygen consumption of the forearm accounted for by glucose utilization (10). The low value of 7 per cent seemed to indicate that oxidation of other substrates was primarily responsible for the oxygen consumption, and it was their suggestion that "the small fraction of fatty acids which is unesterified" may be such an energy source. The majority of Gordon's observations of the arteriovenous differences of total FFA were consistent with this hypothesis (1) but not in all instances. Even though blood was sampled from "deep" veins several negative arteriovenous differences were observed and the presence of these negative arteriovenous differences across the fore-

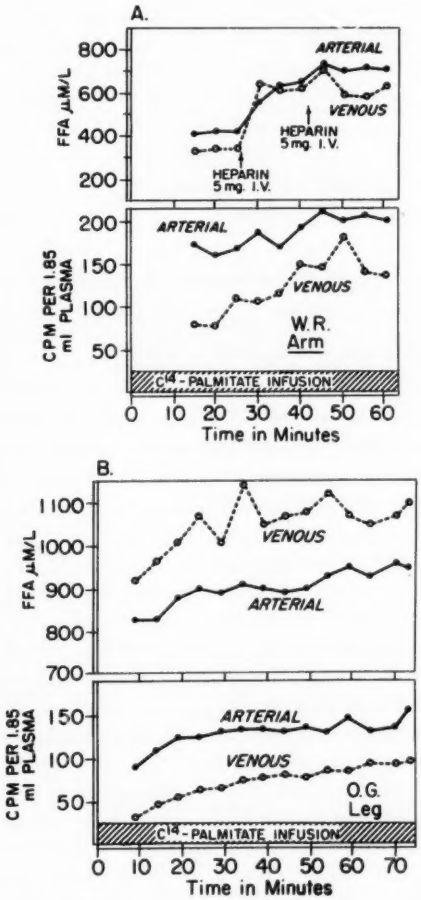

FIG. 1. SIMULTANEOUS ARTERIOVENOUS DIFFERENCES OF PALMITIC ACID-1-C^{14} AND TOTAL PLASMA FREE FATTY ACIDS (FFA) DURING CONSTANT INFUSION OF PALMITIC ACID-1-C^{14}. A. Positive arteriovenous difference of label and positive arteriovenous difference of total FFA. FFA level was raised with heparin to show that behavior of label is independent of FFA concentration. B. Positive arteriovenous difference of label and negative arteriovenous difference of total FFA.

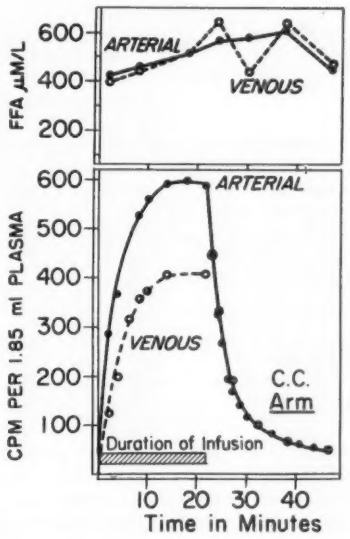

FIG. 2. SIMULTANEOUS ARTERIOVENOUS DIFFERENCES OF PALMITIC ACID-1-C^{14} AND TOTAL PLASMA FFA DURING CONSTANT INFUSION OF PALMITIC ACID-1-C^{14} AND FOLLOWING CESSATION OF INFUSION. Marked uptake of label is shown, while there is no significant difference in venous and arterial total plasma FFA concentration. When infusion is stopped, venous and arterial concentrations of label become almost identical.

TABLE II

Mean equilibrium levels of arterial and venous total plasma free fatty acids and palmitic acid-1-C^{14} during constant infusion of palmitic acid-1-C^{14} *

Subject	Arterial palmitic acid-1-C^{14} activity	Venous palmitic acid-1-C^{14} activity	Arterio-Venous difference of isotope	Arterial FFA conc.	Venous FFA conc.	Arterio-venous difference of FFA
	cpm/1.85 ml plasma		%	*μmoles/L*		%
R.N. leg	170	105	+39	415	475	−14
O.H. leg	130	80	+41	905	1,090	−20
C.D. leg	100	85	+18	540	700	−29
C.C. arm	590	405	+31	480	480	0
E.A. arm	700	430	+38	1,050	830	+21
L.C. arm	740	655	+12	275	305	−12
C.T. arm	890	730	+18	190	210	− 8
H.W. arm	825	735	+11	460	455	0
W.W. arm	240	175	+27	115	125	−11
J.Q. arm	270	155	+44	660	585	+12
J.C. arm	225	185	+19	610	595	+ 2
C.H. arm	290	255	+11	335	355	− 6
F.D. arm	150	95	+35	830	710	+14
C.R. arm	100	80	+16	885	840	+ 5
W.R. arm	225	145	+36	415	335	+20
P.B. arm	90	45	+50	450	440	+ 2

* Values represent means of three or more individual levels.

arm in the resting state properly raised the question of whether or not a negative arteriovenous difference of FFA meant that no FFA was being extracted; Gordon recognized that negative arteriovenous differences in blood sampled from deep forearm veins might indicate simultaneous release of FFA in excess of the fraction removed (1).

The present observations on the arteriovenous differences of infused C^{14}-labeled palmitate add a new dimension to this problem and are interpreted in the following way. In all of our studies, a positive arteriovenous difference was observed, even when the total chemically determined FFA arteriovenous difference was negative. The positive arteriovenous difference of palmitic acid-1-C^{14} is the result of the disappearance of tracer and of endogenous FFA from plasma and the entry of these substances into peripheral tissues, probably mostly muscle. If the studies of Bragdon and Gordon can be applied to man, very little, if any, tracer would be expected to enter fat depots (11). The fact that arteriovenous difference is maintained after prolonged infusion indicates that the arteriovenous difference represents continuous irreversible uptake of palmitic acid-1-C^{14}. In addition, FFA of negligible or zero specific activity from fat depots is added to the venous blood. The effect of this increment of low or zero specific activity may be sufficiently great to produce a negative arteriovenous difference of chemically determined endogenous FFA even though there is a positive arteriovenous difference of palmitic acid-1-C^{14}. At this point interpretation must remain qualitative, and these data cannot be used to measure the amount of FFA extracted. Concerning this qualitative interpretataion, several factors should be pointed out. In these experiments blood was drawn from randomly selected veins. This blood would have perfused regions varying in quantity of fat and muscle, and, therefore, the blood from these regions would also be expected to vary with respect to its composition of chemically determined total plasma FFA and palmitic acid-1-C^{14}. Another factor which could militate against making a quantitative interpretation should be mentioned. Since muscle contains many fat cells, it is conceivable that these cells could add FFA to the blood distal to the point of arterial sampling but proximal to muscle cells so that the blood perfusing these muscle cells might contain a higher concentration of FFA than does the arterial blood. If such an anatomical arrangement exists, the data presented could not be used to quantitate the amount of FFA extracted even if flow rate were known and even if venous blood were homogeneous in concentration. In spite of these variables the results consistently showed a positive arteriovenous difference of label, a difference which was always greater than any posi-

tive arteriovenous difference of chemically determined total plasma FFA. We have concluded, therefore, that in employing the technique of a constant infusion of C^{14}-labeled palmitate, the uptake of fatty acid by peripheral tissue may be adjudged to occur in the presence of evidence of net lipid mobilization from the same peripheral area.

The role which recirculation of label might play in the observed magnitude of the arteriovenous difference of palmitic acid-1-C^{14} is a moot point, since there is no direct evidence that FFA, an insoluble material requiring albumin for its transport, behaves like a freely diffusible ion.

The fate of the removed palmitic acid-1-C^{14} is not answered by these observations. Simultaneous measurement of the arteriovenous difference of labeled $C^{14}O_2$ might provide further information.

SUMMARY AND CONCLUSIONS

1. The arteriovenous difference of palmitic acid-1-C^{14} was measured in the human arm and leg during constant infusion of this isotope. Total plasma free fatty acid arteriovenous differences were measured simultaneously in the same blood.

2. The results indicate that in the resting, fasted subject, palmitic acid-1-C^{14} was extracted by the arm or leg even when there was a net release of total free fatty acids.

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THE INFLUENCE OF ANION PENETRATING ABILITY ON URINARY ACIDIFICATION AND THE EXCRETION OF TITRATABLE ACID *

By NORMAN BANK † and WILLIAM B. SCHWARTZ ‡

(From the Department of Medicine, Tufts University School of Medicine, and the Pratt Clinic-New England Center Hospital, Boston, Mass.)

(Submitted for publication March 3, 1960; accepted May 6, 1960)

Previous studies in the dog have demonstrated that during administration of neutral sodium phosphate the rate of titratable acid excretion is directly proportional to the rate of phosphate excretion and is inversely related to plasma bicarbonate concentration (1). Markedly acid urines containing near theoretical maximum amounts of titratable acid [1] were observed only in the presence of severe metabolic acidosis.

More recently it has been shown that intense acidification of the urine can be achieved without the induction of acidosis (2). Normal subjects stimulated to retain sodium showed a rapid fall in urine pH to approximately 5.0 or below when infused with sodium sulfate, and it was concluded that increased reabsorption of sodium in the presence of a poorly reabsorbable anion was responsible for this phenomenon. Acidification of the urine has also been observed during administration of poorly reabsorbable anions to normal human subjects during quiet standing (3) and to dogs in which glomerular filtration rate was reduced by clamping of the renal artery (4)—experimental procedures known to induce a more complete reabsorption of filtered sodium. These observations raise the possibility that, even without acidosis, large numbers of hydrogen ions might be transported into the glomerular filtrate over a comparably wide pH gradient if a poorly reabsorbable *buffer* anion were infused under appropriate experimental conditions. In the present study it has been demonstrated that, during the infusion of sodium phosphate (pH 7.4) into dogs whose sodium and chloride excretion had been reduced by dietary salt restriction, excretion of titratable acid rises to near theoretical maximum values in the presence of normal plasma bicarbonate concentrations.

These data suggest the hypothesis that a disproportion between the reabsorption of sodium and anion was a critical factor in regulating not only the acidity of the urine but also the rate of excretion of titratable acid. This hypothesis has been further supported by the demonstration that administration of relatively reabsorbable anions, such as chloride or thiocyanate, to phosphate-loaded dogs is associated with a progressive elevation of urine pH and reduction in titratable acid excretion. On the other hand, administration of poorly reabsorbable anions, such as sulfate or ferrocyanide, is accompanied by sustained or increased acidity of the urine and excretion of near theoretical maximum amounts of titratable acid.

As a possible mechanism to account for these findings, it is proposed that the various anions affect urine pH and acid excretion through their influence on the electrical gradient established by active sodium transport across the renal tubular cells. According to this hypothesis, the magnitude of the gradient is determined by the penetrating ability of the available anions, those which penetrate the tubular epithelium more readily having a greater tendency to follow sodium and thus to reduce the potential difference. *Changes* in urinary acidity have tentatively been attributed to the passive movement of hydrogen ions in response to variations in the transtubular electrical gradient.

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‡ Established Investigator of the American Heart Association.

[1] In urines where phosphate is the major buffer, values closely approaching the theoretical maximum for titratable acid are attained when urine pH falls to approximately 5.5, since at this point more than 95 per cent of the phosphate (pK_2' approximately 6.8) is in the $H_2PO_4^-$ form.

Although it seems probable that active transport of hydrogen is also involved in the over-all acidifying process, the present data suggest that passive diffusion of hydrogen plays an important role in the mechanism governing urine acidification and the renal excretion of titratable acid.

METHODS

Twenty-one experiments were performed on 21 female mongrel dogs, varying in weight from 10 to 20 kg. The animals were fed 100 g of ground horse meat for 10 to 14 days prior to the day of the experiment. This diet contained approximately 4 mEq Na^+, 8 mEq Cl^-, and 6 mEq K^+ daily. Sixty to 90 minutes before each experiment, 500 ml of water was administered through a gastric tube. Anesthesia was induced by intravenous administration of chloralose, 100 mg per kg, as a 1.5 per cent solution in 2 per cent dextrose. Additional amounts were administered during the course of the experiment as required.

Each experiment was begun with the intravenous infusion of 4 per cent dextrose in water at a rate of 2.4 to 6.0 ml per minute. After an adequate urine flow had been established (approximately 3 to 6 ml per minute), two control urine collections of 10 to 15 minutes each were obtained. The dextrose solution was then replaced by one containing 60 mmoles per L of sodium phosphate (48 mmoles $HPO_4^=$, 12 mmoles $H_2PO_4^-$) delivered at 4.2 ml per minute. After collecting urine for four to seven periods of 10 to 15 minutes each, this solution was replaced by one of the following, in millimoles per liter: NaCl, 108 (9 experiments); Na_2SO_4, 54 (4 experiments); NaSCN, 108 (4 experiments); and $Na_4Fe(CN)_6$, 27 (4 experiments); each was delivered at 4.2 ml per minute. The concentration of sodium and its rate of administration was thus the same during sodium phosphate loading and during subsequent loading with other salts. In order to maintain the osmolarity of all solutions at about 220, appropriate amounts of dextrose were added to the Na_2SO_4 and $Na_4Fe(CN)_6$ solutions.

Urines were collected through an indwelling bladder catheter and the bladder was emptied by manual compression at the end of each period. pH was determined on aliquots collected anaerobically at the midpoint of each period. Heparinized arterial blood samples were collected for determination of pH, total CO_2, chloride and potassium.

Urine and blood pH were determined anaerobically at 37° C with a Cambridge model R pH meter. CO_2 contents were determined manometrically by the method of Peters and Van Slyke (5). Urine sodium and potassium were measured by a Baird flame photometer using an internal lithium standard. Urine phosphorus was determined by the method of Fiske and Subbarow (6). Chloride was measured in urine and plasma by titration with silver nitrate, using a potentiometric method (7). Urine ferrocyanide was measured by the method of Berliner, Kennedy and Hilton (8), ammonium by the method of Folin and Bell (9), and inorganic sulfate was determined gravimetrically as barium sulfate (5). Thiocyanate was measured in urine and plasma by the method of Barker (10). Chloride was calculated for specimens containing thiocyanate by subtracting the measured thiocyanate concentration from the sum of chloride and thiocyanate as determined by titration with $AgNO_3$. In specimens containing ferrocyanide, chloride and phosphate were determined after removal of the ferrocyanide by precipitation with $ZnSO_4$.

The value for titratable acid was calculated from the urine phosphate, urine pH and blood pH.[2] The pK_2' of phosphate in blood was taken to be 6.8. The same value was used arbitrarily for urine when urine pH was below 6.0, since the error introduced into the calculation of titratable acid is less than 5 per cent even when pK_2' is as much as ± 0.2 unit different from 6.8 (11). The pK_2' was calculated for individual specimens with pH values above 6.0, since in such urines appreciable errors in estimation of titratable acid may result from use of an incorrect pK_2'. On these specimens additional analyses were made for CO_2 and organic acids (11) in order to make a closer approximation of ionic strength. The theoretical considerations and method of calculation of pK_2' from ionic strength have been described in a previous publication from this laboratory (11).

For each urine specimen, titratable acid has been expressed as a percentage of the theoretical maximum titratable acid. This latter value was calculated by subtracting from total phosphate excretion that amount already present as $H_2PO_4^-$ at the pH of the plasma (and filtrate). For example, when plasma pH is 7.4 and 20 per cent of the phosphate is thus present as $H_2PO_4^-$, the value for theoretical maximum titratable acid is equivalent to 80 per cent of total urine phosphate.

RESULTS

A. Effect of sodium phosphate infusion on urine pH and titratable acid excretion. Tables I to IV show the results of four representative experiments. During the control periods urine pH ranged between 6.32 and 6.83, chloride excretion ranged between 8 and 27 μmoles per minute, and phosphate excretion was 6 μmoles per minute or less. With the infusion of sodium phosphate, phosphate excretion rose to between 149 and 186 μmoles per minute and titratable acid excretion to between 102 and 143 μEq per minute. In all but one of the experimental periods, titratable acid

[2] The contribution of organic acids to titratable acid excretion was found to be negligible by potentiometric titration of urine from which phosphate had been removed by calcium hydroxide precipitation. The titration was carried out between the original pH of the urine and the pH of the plasma.

ranged between 87 and 99 per cent of the theoretical maximum. Urine pH in each instance fell within 15 minutes to values approximately 0.5 to 1.0 unit below those of the control periods and, as phosphate excretion increased, fell further to a level which ranged between 4.43 and 5.80. Chloride excretion fell in each case from control levels to less than 10 μmoles per minute and sodium excretion rose progressively.

Blood pH ranged between 7.25 and 7.39 during control periods and from 7.26 to 7.35 during phosphate loading. Bicarbonate concentrations were between 18.5 and 24.2 mmoles per L during control periods and between 18.5 and 22.2 during phosphate loading; P_{CO_2} was between 37 and 51 mm Hg during control periods and between 37 and 47 during phosphate loading.

The results of 17 experiments in which sodium phosphate was infused initially and NaCl, Na_2SO_4 or $Na_4Fe(CN)_6$ was given subsequently, are shown in Figures 1 and 2. The periods of sodium phosphate infusion are shown in each figure as solid circles. The other symbols, referring to a latter portion of each study, will be discussed separately. Phosphate excretion is plotted on the abscissa and the percentage of theoretical maximum titratable acid on the ordinate. Two points shown for convenience at 210 μmoles per minute were actually 221 and 255. It is clear that, during infusion of sodium phosphate, titratable acid excretion was close to the theoretical maximum over the entire range of phosphate excretion. The absolute values for titratable acid excretion rose to a maximum of 195 μEq per minute during phosphate infusion. Urine pH during control periods was usually between 6.0 and 7.0 and with the infusion of sodium phosphate fell to levels between 4.4 and 6.0. The average chloride excretion during control periods was 10 μmoles per minute, with a range from 1 to 28. During phosphate loading chloride excretion fell to an average of 4 μmoles per minute, with a range of 1 to 12. There were usually small but significant increases in excretion of both potassium and ammonium during phosphate loading.

Plasma bicarbonate concentration was between 18 and 22 mmoles per L in almost all experiments, and blood pH ranged between 7.25 and 7.44. In the individual experiments, blood pH and total CO_2 changed only slightly during control and phosphate loading periods.

B. Effect of NaCl infusion on urine pH and titratable acid excretion. In Table I are shown the results of a typical experiment in which NaCl was given following a sodium phosphate infusion. With the administration of NaCl, titratable acid excretion fell from 99 per cent of theoretical maximum to 42 per cent. Urine pH rose abruptly from 4.71 to 6.41 and continued to rise to 6.80 as chloride was infused. Chloride excretion rose progressively. Phosphate excretion fell gradually

TABLE I

The effect of a sodium phosphate infusion and a subsequent infusion of sodium chloride on urine pH and titratable acid (T.A.) excretion

	Urine										Plasma			
Time	Na	K	NH_4	Cl	P	pH	pK_1'	T.A.	Theor. max. T.A.	Max. T.A.	pH	HCO_3	P_{CO_2}	Cl
min	*μmoles/min*							*μEq/min*		*%*		*mmoles/L*	*mmHg*	*mEq/L*
0	Infusion: dextrose, 222 mmoles/L at 4.2 ml/min										7.39	21.7	37	106
50–65	11	28	29	12	5	6.32								
65–80	43	36	28	27	5	6.79					7.34	21.5	41	95
85	Infusion: Na_2HPO_4, 48; NaH_2PO_4, 12; dextrose, 110 mmoles/L; at 4.2 ml/min													
95–110	1	19	32	2	20	5.67	6.80	14	15.5	90				
110–125	8	43	41	2	58	4.59	6.80	44	45	98	7.35	20.4	38	93
125–140	43	59	45	3	109	4.43	6.80	84	85	99				
140–155	95	55	34	4	144	4.96	6.80	110	112	98				
155–170	118	49	31	5	153	5.40	6.80	113	119	95	7.33	22.2	43	93
170–185	117	48	32	5	157	5.42	6.80	116	122	95				
185–200	141	63	36	5	186	4.71	6.80	143	144	99	7.33	22.1	43	91
202	Infusion: NaCl, 108 mmoles/L, at 4.2 ml/min													
215–230	181	37	24	15	149	6.41	6.84	76	116	66				
230–245	212	42	22	32	124	6.60	6.86	52	96	54	7.34	23.7	45	95
245–260	184	38	21	26	101	6.65	6.87	40	78	51				
260–275	173	33	21	28	80	6.80	6.88	26	62	42	7.35	23.5	44	98

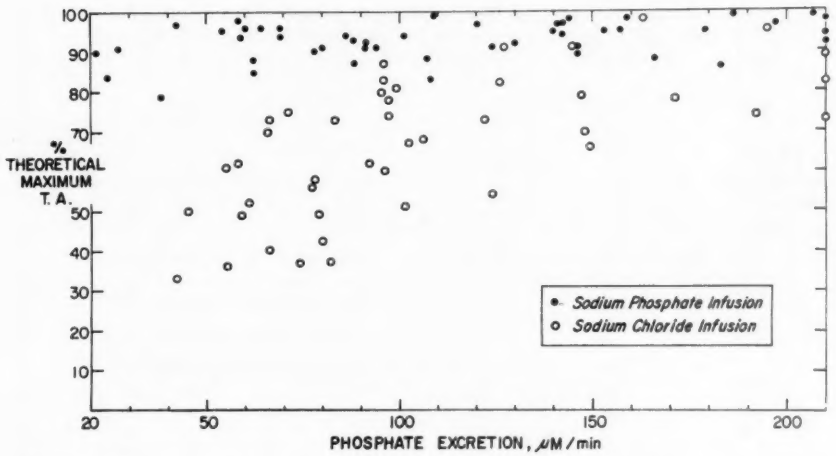

FIG. 1. THE EFFECT OF SODIUM PHOSPHATE INFUSION AND A SUBSEQUENT INFUSION OF SODIUM CHLORIDE ON THE PERCENTAGE OF THEORETICAL MAXIMUM TITRATABLE ACID EXCRETION. For definition of "theoretical maximum titratable acid," see text.

during the next hour but the excretion rates were comparable with those of the preceding phosphate loading periods. Sodium excretion was higher than during the previous periods of phosphate loading.

The highest blood pH was 7.35, a value essentially the same as that during earlier periods when urine pH was between 4.43 and 5.67. Plasma bicarbonate rose about 1.5 mmoles per L and P_{CO_2} showed no significant change. Plasma chloride concentration rose 7 mEq per L.

The results of all nine experiments are shown in Figure 1 in which the NaCl periods are represented by open circles. The percentage of maximum titratable acid excretion fell progressively to levels well below those seen at comparable rates of phosphate excretion during sodium phosphate administration. Urine pH usually rose to above 6.0

TABLE II

The effect of a sodium phosphate infusion and a subsequent infusion of sodium sulfate on urine pH and titratable acid excretion

	Urine								Theor. max.	Max.	Plasma			
Time	Na	K	NH_4	Cl	SO_4	P	pH	T.A.	T.A.	T.A.	pH	HCO_3	P_{CO_2}	Cl
min				*μmoles/min*				*μEq/min*		*%*		*mmoles/L*	*mmHg*	*mEq/L*
0	Infusion: dextrose, 222 mmoles/L, at 4.2 ml/min										7.30	24.2	51	100
80–95	112	45	26	23		6	6.83							
95–110	44	30	28	17		3	6.69				7.32	20.3	41	88
115	Infusion: Na_2HPO_4, 48; NaH_2PO_4, 12; dextrose, 55 mmoles/L; at 4.2 ml/min													
125–140	6	43	37	3		42	5.67	30	33	91				
140–150	18	69	37	2		79	5.38	58	62	94				
150–160	36	77	35	2		105	5.23	79	82	96	7.35	20.0	37	88
160–170	63	79	36	2		132	5.22	99	103	96				
170–180	85	75	38	3		149	5.18	113	116	97				
180–192	92	82	38	2		161	5.28	123	128	96				
195	Infusion: Na_2SO_4, 54; dextrose, 55 mmoles/L; at 4.2 ml/min													
205–215	139	80	39	4	44	144	5.62	105	115	91	7.39	20.9	36	88
215–225	158	79	45	3	68	118	5.12	91	94	97				
225–235	168	72	46	3	72	101	5.04	79	80	99				
235–245	202	76	41	3	83	99	5.11	77	79	98	7.40	22.9	38	88
245–255	218	76	41	3	116	97	5.10	76	77	99				

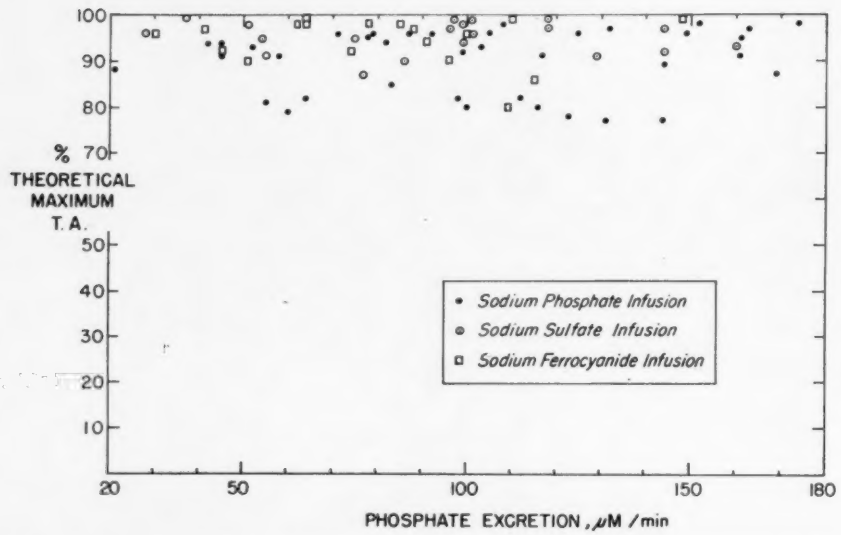

FIG. 2. THE EFFECT OF SODIUM PHOSPHATE INFUSION AND A SUBSEQUENT INFUSION OF SODIUM SULFATE OR SODIUM FERROCYANIDE ON THE PERCENTAGE OF THEORETICAL MAXIMUM TITRATABLE ACID EXCRETION.

shortly after NaCl was begun and continued to rise during the next 40 to 60 minutes to levels between 6.5 and 7.0 as chloride excretion increased from 15 to 130 μmoles per minute. In a few instances, a significant rise in urine pH and fall in titratable acid did not occur until the second period after beginning NaCl administration. Although it is not apparent from the figure, a close correlation existed between the first appearance of increased quantities of chloride in the urine and both the rise in pH and the fall in percentage of theoretical maximum titratable acid.[3]

Blood pH during NaCl infusion ranged between 7.30 and 7.44, and plasma bicarbonate between 17 and 25 mmoles per L for the group. Changes in these values between the sodium phosphate and sodium chloride periods were small and variable.

C. Effect of Na_2SO_4 infusion on urine pH and titratable acid excretion. In Table II are shown the results of a typical experiment in which Na_2SO_4 was given following a sodium phosphate infusion. It is apparent that, during Na_2SO_4 administration, titratable acid excretion remained at over 90 per cent of the theoretical maximum, and there was a slight further fall in urine pH. Chloride excretion was 2 to 3 μmoles per minute during phosphate administration and showed no significant change during sulfate infusion. Sodium excretion rose progressively throughout the entire experiment.

Blood pH, bicarbonate and chloride varied only slightly between the phosphate and sulfate infusion periods.

The results of all four experiments are shown in Figure 2, in which the open circles represent periods during Na_2SO_4 infusion. The infusion of this salt was accompanied by excretion of near theoretical maximum amounts of titratable acid at rates of phosphate excretion comparable with those during sodium phosphate infusion. In three of the four experiments urine pH fell to lower levels than those achieved during phosphate infusion. In the fourth, there was no significant change from the acid values achieved during the phosphate periods. Chloride excretion was below 10 μmoles per minute during sulfate loading. Blood pH ranged between 7.30 and 7.40, plasma bicarbonate between

[3] In two experiments (not shown) initial acidification of the urine occurred with administration of phosphate but was followed by a sudden spontaneous rise in chloride excretion and pH, possibly due to an osmotic effect of the phosphate load. In a few additional experiments, where control chloride excretion was high (100 to 200 μEq per minute), little or no reduction in urine pH occured during phosphate infusion.

TABLE III

The effect of a sodium phosphate infusion and subsequent infusions of sodium ferrocyanide and sodium chloride on urine pH and titratable acid excretion

Time	Urine Na	K	NH_4	Cl	$Fe(CN)_6$	P	pH	T.A.	Theor. max. T.A.	Max. T.A.	Plasma pH	HCO_3	P_{CO_2}	Cl
min	*μmoles/min*							*μEq/min*		%		*mmoles/L*	*mmHg*	*mEq/L*
0	Infusion: dextrose, 222 mmoles/L at 4.2 ml/min										7.27	20.3	46	105
45–60	33	24	41	25	0	4	6.50							
60–70	40	24	42	17		4	6.44				7.25	18.5	44	98
71	Infusion: Na_2HPO_4, 48; NaH_2PO_4, 12; dextrose, 55 mmoles/L; at 4.2 ml/min													
80–90	76	34	45	9		82	5.49	58	62	94				
90–100	115	35	42	7		125	5.34	90	94	96				
100–110	149	36	47	5		163	5.32	118	122	97	7.27	18.5	42	99
110–120	174	29	31	6		169	5.80	111	127	87				
122	Infusion: $Na_4Fe(CN)_6$, 27; dextrose, 83 mmoles/L; at 4.2 ml/min													
130–140	275	50	36	8	29	148	4.62	110	111	99				
140–150	373	53	34	8	54	110	4.44	82	83	99	7.28	18.2	40	95
150–160	399	50	32	6	63	85	4.45	63	64	98				
160–170	476	57	34	8	78	78	4.65	59	60	98				
172	Infusion: NaCl, 108 mmoles/L at 4.2 ml/min													
180–190	445	64	26	26	62	43	6.37	21	33	64	7.31	19.4	40	95
190–200	413	52	26	43	50	26	6.75	8	20	40				
200–210	308	41	26	43	35	15	6.84	4	11	36				
210–220	289	41	27	62	33	15	6.81	4	11	36	7.30	18.2	38	110

16 and 23 mmoles per L and P_{CO_2} between 30 and 40 mm Hg. These values were not significantly different from those of the preceding phosphate loading periods.

D. Effect of $Na_4Fe(CN)_6$ infusion on urine pH and titratable acid excretion. In Table III are shown the results of a typical experiment in which sodium ferrocyanide was given following a sodium phosphate infusion. The infusion of $Na_4Fe(CN)_6$ was accompanied by a slight rise in the percentage of theoretical maximum titratable acid to values of 98 to 99 per cent. Urine pH fell, from a range of 5.32 to 5.80, to 4.44 to 4.65, and chloride excretion remained below 10 μmoles per minute. During the last four periods, sodium ferrocyanide was replaced by sodium chloride. There was a prompt fall in the percentage of theoretical maximum titratable acid.[4] Urine pH rose from 4.65 to 6.37 in the first period and continued to rise to 6.84. Ferrocyanide excretion continued in significant amounts and chloride excretion rose progressively. Sodium excretion increased during ferrocyanide infusion and decreased during the final periods of sodium chloride infusion.

Blood pH, plasma bicarbonate and P_{CO_2} remained essentially constant during the periods of both phosphate and ferrocyanide administration. Plasma chloride concentration was unchanged during ferrocyanide infusion and rose to 110 mEq per L when NaCl was administered.

The results of all four experiments are shown in Figure 2 in which the open squares represent the periods of $Na_4Fe(CN)_6$ infusion. It is evident that titratable acid excretion was more than 90 per cent of theoretical maximum at rates of phosphate excretion comparable with those during sodium phosphate infusion. Urine pH values in each instance were lower than those achieved during the previous phosphate infusion periods. Chloride excretion remained below 10 μmoles per minute during ferrocyanide infusion.

Blood pH ranged between 7.28 and 7.44, plasma bicarbonate between 17 and 22 mmoles per L and P_{CO_2} between 28 and 47 mm Hg during sodium ferrocyanide infusion. There were no significant changes from the values of the preceding phosphate loading periods.

E. Effect of NaSCN infusion on urine pH and titratable acid excretion. In Table IV are shown the results of the experiment in which occurred the most prompt response after starting sodium thiocyanate infusion. Urine pH rose from 5.74 to 6.62 and titratable acid excretion fell from 90

[4] Since the specific effects of ferrocyanide on the pK_2' of phosphate are not known, a value of 6.8 has been used arbitrarily.

TABLE IV

The effect of a sodium phosphate infusion and a subsequent infusion of sodium thiocyanate on urine pH and titratable acid excretion

	Urine									Theor. max.	Max.	Plasma			
Time	Na	K	NH_4	Cl	SCN	P	pH	pK_1'	T.A.	T.A.	T.A.	pH	HCO_3	Pco_2	Cl
min			*μmoles/min*						*μEq/min*		%		*mmoles/L*	*mmHg*	*mEq/L*
0	Infusion: dextrose, 222 mmoles/L at 4.2 ml/min											7.28	22.2	49	99
65–80	10	21	27	12		1	6.75								
80–95	7	17	28	8		1	6.60					7.26	20.7	48	93
120	Infusion: Na_2HPO_4, 48; NaH_2PO_4, 12; dextrose, 55 mmoles/L; at 4.2 ml/min														
130–140	33	36	38	8		53	6.16		30	39	77				
140–155	26	24	24	2		50	5.58	6.80	34	37	92	7.26	20.3	47	91
170–185	104	44	28	4		130	5.72	6.80	87	97	90				
185–200	122	48	36	4		146	5.68	6.80	98	108	91				
200–215	144	49	33	5		149	5.74	6.80	102	114	90				
220	Infusion: NaSCN 108 mmoles/L at 4.2 ml/min														
235–250	231	47	21	8	1	165	6.62	6.80	60	126	48	7.32	21.3	43	86
250–265	164	33	20	8	1	105	6.67	6.87	40	80	50				
265–275	210	42	23	22	2	107	6.79	6.91	35	82	43				
275–285	286	59	28	70	5	107	6.85	6.90	31	82	38	7.30	21.4	45	83

to 48 per cent of theoretical maximum within 20 minutes. Thiocyanate excretion rose to 5 and chloride excretion from 5 to 70 μmoles per minute. A similar increase in chloride excretion following administration of thiocyanate has previously been noted by Rapoport and West (12).

Blood pH, plasma bicarbonate and Pco_2 remained within a normal range throughout the experiment.

In two of the other three experiments, there was a fall in titratable acid excretion from over 90 to less than 60 per cent of theoretical maximum and a progressive rise in urine pH from 5.56 and 5.28 to 6.58 and 6.57, respectively. However, these changes took place more slowly than in the experiments in which chloride was administered, and the maximal changes occurred only after 40 to 60 minutes of thiocyanate infusion. Furthermore, they were accompanied by only a slight rise in chloride and thiocyanate excretion. It is probable that this delay was due to almost complete reabsorption of thiocyanate and chloride proximal to the acidifying site. In the fourth experiment the changes in urine pH and titratable acid were small. At the end of 60 minutes pH had risen only from 5.44 to 5.98, and titratable acid excretion had fallen from 93 to 81 per cent of theoretical maximum. However, it should be noted that no thiocyanate appeared in the urine during this time, and there was no rise in chloride excretion.

In the latter three experiments, blood pH ranged between 7.25 and 7.35, plasma bicarbonate between 17 and 21 mmoles per L and Pco_2 between 34 and 49 mm Hg during NaSCN infusion. There were no changes from the values of the preceding sodium phosphate periods.

DISCUSSION

The present experiments demonstrate that administration of neutral sodium phosphate to dogs whose sodium chloride excretion has been reduced by dietary salt restriction is associated with a prompt and marked fall in urine pH and excretion of near theoretical maximum amounts of titratable acid. Titratable acid excretion remains close to these maximal values despite a progressive increase in phosphate excretion to as much as 250 μmoles per minute. The results indicate that, over the range of phosphate excretion rates which was studied, acidosis is not necessary for excretion of maximal amounts of acid. These observations are in accord with the view that a disproportion between sodium and anion reabsorption was the critical factor in determining not only the hydrogen ion gradient between blood and urine but also the total excretion of acid. This view is supported by the finding that the percentage of theoretical maximum titratable acid is decreased by the infusion of the readily reabsorbable anions, chloride and thiocyanate, and is sustained or increased by infusion of the poorly reabsorable anions, sulfate or ferrocyanide.

No complete explanation for the influence of various anions on the process of acid excretion will be possible until more detailed information is available concerning the forces governing ion transfers across the renal tubule. However, it is of considerable interest that the results of the present experiments may readily be accounted for if it is assumed that the mechanism by which the anions affected acid excretion operated through their influence upon the electrical gradient across the renal tubular cells (13, 14). Active transport of sodium [which has recently been demonstrated for the renal tubule (15)] has been shown to account fully for the electrical potential difference (P.D.) across a variety of membranes (16, 17). The magnitude of the P.D. in such membranes depends upon both the rate at which sodium is transported and the resistance of the membrane to the ions that distribute themselves passively and which, by their diffusion, tend to reduce the P.D. (18, 19). In these terms the data in the present experiments might be explained in the following fashion: when sodium phosphate is infused into animals excreting little chloride, sodium reabsorption is increased at a time when only a relatively nonpenetrating anion, phosphate, is present in significant quantities. As a consequence, the P.D. across the tubular cell increases, the lumen becoming more negative, and H^+ moves passively along the electrical gradient into the lumen, titrating the phosphate and reducing the urine pH.[5] According to this hypothesis, not only phosphate but other anions in the filtrate should also influence the P.D. and, therefore, the acidifying process, in a manner dictated by their ability to penetrate the tubular epithelium. This "penetrating ability" has been shown by Rapoport and West (12) to follow closely the lyotropic series ($SCN^- > Cl^- > HPO_4^= > SO_4^= > Fe(CN)_6^=$) in a fashion similar to that observed for red cells, unicellular organisms, plant cells, and intestine (20, 21). Thus, when anions of low penetrating ability such as sulfate or ferrocyanide are infused, they sustain or increase the P.D. and the transfer of H^+ to the filtrate. On the other hand, it is postulated that anions such as chloride or thiocyanate,[6] which can move readily along the electrical gradient set up by active sodium transport, decrease the P.D. and reduce passive movement of hydrogen into the lumen even when large amounts of phosphate are present. This hypothesis is supported by the demonstration that the potential difference established across isolated frog skin by active sodium transport is much greater when a nonpenetrating anion such as sulfate is substituted in the bathing solution for a relatively penetrating anion such as chloride (19).

In the preceding discussion *changes* in pH and acid excretion have been described in terms of passive movement of hydrogen ions in response to a potential difference established across the renal tubule by active sodium transport. This view in no way excludes the possibility that active hydrogen transport plays an important role in the overall acidifying process but does suggest that anion penetrating ability can exert a critical influence on the mechanism governing urinary pH and the renal excretion of acid.

It is not meant to imply that the presence of large amounts of chloride in the urine is necessarily incompatible with aciduria. It is well known, for example, that during the chloruresis associated with ammonium chloride acidosis urine pH is low (23), and it seems probable that other factors such as a low intracellular pH, a reduced cellular potassium content, or a change in the permeability of the tubular epithelium to chloride might account for these findings. In this regard, it is of interest that permeability of the isolated frog skin to chloride may vary widely, e.g., when trace amounts of copper (10^{-5} M) are added to the bathing solution, chloride becomes virtually nonpenetrating (19). Under these circumstances potential differences of 120 to 130 mv develop across the membrane. Whether a comparable change in permeability as a consequence of metabolic acidosis might account for urinary acidification in the presence of chloruresis, or whether

[5] The simultaneous increase in potassium excretion observed in these experiments could also be accounted for by the movement of intracellular potassium into the filtrate in response to the increased negativity of the lumen.

[6] It should be noted that thiocyanate inhibits acid secretion by the gastric mucosa without producing a significant change in electrical potential (22). However, in view of the striking differences between the mechanisms of gastric and renal acid formation, it seems improbable that the mode of action of thiocyanate on stomach and kidney is the same.

some other factors are responsible, remains to be determined.

SUMMARY

The influence on acid excretion of anions differing in penetrating ability has been studied in dogs whose sodium and chloride excretion had been reduced by dietary restriction. It was demonstrated that administration of the sodium salt of phosphate, a poorly reabsorbable buffer anion, leads to a marked reduction in urine pH and the excretion of near theoretical maximum amounts of titratable acid. These results indicate that, over the range of phosphate excretion rates which has been studied, maximal excretion of acid can be achieved without the stimulus of acidosis. It has been suggested that a disproportion between sodium and anion reabsorption was the critical factor in determining not only the pH gradient between blood and urine but also the total excretion of acid. This hypothesis is supported by the finding that the percentage of theoretical maximum titratable acid excreted in the urine is decreased by the subsequent infusion of the readily reabsorbable anions, chloride or thiocyanate, and is sustained or increased by the administration of the poorly reabsorbable anions, sulfate or ferrocyanide.

As a possible mechanism to account for these findings, it is proposed that the various anions affected urine pH and acid excretion through their influence on the electrical gradient established by active sodium transport across the renal tubular cells. According to this hypothesis, the magnitude of the gradient is determined by the relative penetrating ability of the available anions, those which penetrate the tubular epithelium more readily having a greater tendency to follow sodium and thus to reduce the potential difference. Changes in urinary pH and titratable acid excretion might thus be attributed to the passive movement of hydrogen ions in response to variations in the transtubular electrical gradient. This view in no way excludes the possibility that active hydrogen transport plays an important role in the over-all acidifying process but does suggest that anion penetrating ability can exert a critical influence on the mechanism governing urinary pH and the renal excretion of acid.

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THE ROLE OF LACTIC ACID IN THE REDUCED EXCRETION OF URIC ACID IN TOXEMIA OF PREGNANCY *

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When Chesley and Williams demonstrated low uric acid excretion in toxemic patients,[1] they noted that the reduction in renal urate clearance was relatively greater than the reduction in glomerular filtration rate (1). This relationship has been confirmed (2) and is striking when compared with observations on urate excretion in normal subjects and patients with renal disease. It is generally accepted that under normal circumstances serum urate is completely filtered at the glomerulus and that 5 to 10 per cent of filtered urate appears in the urine (3); net tubular reabsorption accounts for the other 90 to 95 per cent. Coombs and co-workers (4), in a study of subjects with various degrees of renal impairment, found that as glomerular filtration rate fell, a progressively larger portion of filtered urate was excreted in the urine, uric acid clearance increasing to over 20 per cent of glomerular filtration rate. In pre-eclamptic and eclamptic patients, although glomerular filtration rate is reduced, the ratio of urate clearance to glomerular filtration rate is often lower than normal. Unless there is a reduction in the filtrability of serum urate, explanation of the low ratio of uric acid clearance to glomerular filtration rate must invoke greater net tubular reabsorption of uric acid in these patients.

The ingestion of buffered lactic acid is normally folowed by reduced uric acid excretion (5). Quick confirmed this response to lactate (6) and suggested that lactate might be responsible for the hyperuricemia of pre-eclampsia and eclampsia (7). At that time there were a few reports in the literature of high blood concentrations of lactic acid in toxemic patients (8–10). However, Schaffer, Barker, Summerson and Stander (11) were unable to find a correlation between *blood levels* of uric acid and lactic acid in each patient with pre-eclampsia, although the mean concentration of each metabolite was higher in the group with pre-eclampsia than in their normal pregnancy group. More recently, Huckabee (12) has shown the importance of measuring lactic acid in arterial rather than in venous blood, as well as other refinements in technique.[2] Thus, failure to relate blood lactic acid concentration to hyperuricemia in toxemic patients may have been due to methodological deficiencies.

This study was initiated to re-evaluate the role of lactic acid in the reduced urate clearance of toxemia of late pregnancy. The results indicate an inverse relationship between arterial lactic acid concentration and uric acid excretion in normal and toxemic pregnancy.

MATERIAL AND METHODS

Twenty-eight patients were studied in the last trimester of pregnancy, before the onset of labor. All of the patients were Negro except N.M. and P.Z. Ten subjects were in a normal pregnancy. Edema, proteinuria, and a rise in blood pressure, or 2 of these abnormalities, were present in 11 patients classified as pre-eclamptic. Three patients with eclampsia had the aforementioned abnormalities; in addition, they convulsed. Four subjects who

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[1] The term "toxemia of late pregnancy" in this paper is intended to describe patients who manifest albuminuria, hypertension, and edema in the latter half of pregnancy. Since classification of patients into the categories of chronic renal disease, chronic hypertension, or pre-eclampsia is so often difficult, and is unnecessary for the purposes of this paper, toxemia will be used interchangeably with pre-eclampsia and eclampsia.

[2] The author is grateful to Dr. W. E. Huckabee for valuable suggestions regarding the measurement of lactic acid in blood.

were known to have hypertension very early in pregnancy as well as at the time of study, but who had no other abnormalities, were considered to have chronic hypertensive vascular disease. The length of the pregnancy in each case was based upon the date of the last normal menstrual period unless this was considered incorrect for other reasons.

All subjects were examined in the resting, hydrated, postabsorptive state, and the 3 eclamptic patients at least 12 hours after convulsing. A minimum of 1 hour was allowed to elapse between insertion of the Cournand arterial needle, used for blood sampling, and the start of collection periods. A urethral catheter was used to facilitate urine collection. Uric acid clearance (C_{urate}) and endogenous creatinine chromogen clearance (C_{cr}) were determined for 3 consecutive 15-minute periods. Blood samples were obtained midway in each collection period. Arterial whole blood specimens for lactic acid were collected by allowing blood to flow directly into a small chilled tube and immediately transferring 1 ml of blood into 10 ml of 10 per cent trichloroacetic acid in an ice bath. Specimens were shaken frequently and allowed to stand at 0°C for at least 2 hours. The precipitate was separated by centrifugation, and the supernatant fluid was run in triplicate using the method of Barker and Summerson (13). Recovery of lactic acid added to the trichloroacetic acid just before the blood was 100 per cent ± 5 per cent. Lactic acid concentrations are reported as milligrams of lactate per 100 ml of whole blood. According to Huckabee (12), the ratio of the lactate concentration in whole blood water to the lactate concentration in plasma water averages 1.08. Uric acid and creatinine were measured by the method of Praetorius and Poulsen (14), and Bonsnes and Taussky (15), respectively.

The effect of lactate on a normal pregnant subject near term was tested by an intravenous infusion of isotonic sodium lactate, 15 ml per minute for 45 minutes.

RESULTS

The response of four normal pregnant subjects near term to intravenous sodium lactate is listed in Table I. Lactate was infused after three control periods and was associated with a marked fall in urate clearance. There was no consistent change in creatinine clearance. Variations in the latter are probably due to collection errors which

TABLE I

The response of four normal pregnant subjects to an intravenous infusion of sodium lactate *

Patient	Month of pregnancy	Serum creatinine	Serum uric acid	C_{urate}	C_{cr}	$C_{urate}:C_{cr}$
		mg/100 ml	*mg/100 ml*	*ml/min*	*ml/min*	%
M.G.	9	0.78	4.9	7.6	103	7.4
				10.0	112	8.9
				13.8	139	10.0
				3.9	69	5.7
				1.9	86	2.2
				1.4	86	1.6
H.P.	8	0.60	2.5	5.2	65	8.0
				6.4	75	8.5
				5.6	70	8.0
				4.8	62	7.7
				1.8	73	2.5
				1.6	58	2.8
S.R.	8	0.73	4.9	12.4	148	8.4
				8.4	104	8.1
				13.4	150	8.9
				6.9	86	8.0
				4.1	125	3.3
				3.3	125	2.6
C.W.	9	0.69	4.1	9.4	112	8.4
				8.4	106	7.9
				12.0	142	8.5
				8.5	119	7.1
				4.5	174	2.6
				2.0	109	1.8

* The first three clearance periods for each patient were control observations. The lactate infusion was started and continued as the next three 15-minute collection periods were obtained. Abbreviations used: C_{urate} = clearance of urate; C_{cr} = clearance of creatinine; $C_{urate}:C_{cr}$ = ratio of C_{urate} to $C_{cr} \times 100$.

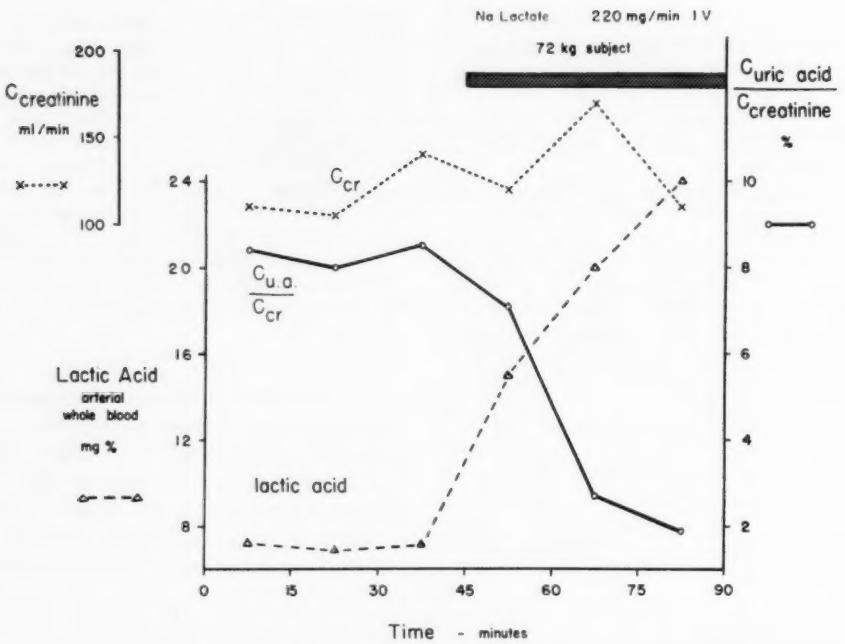

FIG. 1. RESPONSE OF A NORMAL PREGNANT SUBJECT TO AN INTRAVENOUS INFUSION OF ISOTONIC SODIUM LACTATE. Lactate infusion started at 45 minutes. As arterial lactic acid concentration rose, the ratio of uric acid clearance to creatinine clearance fell sharply.

would affect the simultaneous urate clearance in a similar manner. Arterial lactate concentrations were measured in Patient C.W. whose response is illustrated in Figure 1.

The clinical diagnosis, average resting endogenous arterial lactate concentration, and ratio $C_{urate}:C_{cr}$ for each patient are listed in Table II and plotted in Figure 2. There was a significant ($p < 0.005$) negative correlation ($R = -0.60$) between arterial lactate levels and the ratio $C_{urate}:C_{cr}$; i.e., the higher concentrations of whole blood lactate were associated with the lower ratios of urate clearance to creatinine clearance. If the three eclamptic patients are omitted from the analysis, a similar correlation is found ($R = -0.50$, $p < 0.05$). However, there was no correlation between arterial lactate concentrations and the clearance ratio in the normal subjects.

Four patients with low clearance ratios were restudied on their third or fourth postpartum day. In each case, abnormal prenatal lactate concentrations and clearance ratios had returned to the normal range (Table III, Figure 3). Patient N.M. had an abnormal antepartum clearance ratio which returned to normal in the postpartum period, although there was no significant change in her blood lactate concentration, which had not been elevated.

DISCUSSION

There are now several reports that blood lactic acid concentrations are higher in patients with toxemia than in patients during normal pregnancy (8–10, 16, 17). Differing methodology probably explains the differences in conclusions as to lactate concentrations in normal pregnancy. Arterial lactic acid concentrations in normal pregnant subjects in this study were in a range similar to those found in resting nonpregnant subjects by Huckabee (12) but were generally lower than the values reported by Hendricks (18) who measured the lactic acid content of maternal venous blood.

The reduction in the uric acid clearance of normal pregnant subjects in response to lactate infusion was similar to that observed in nonpregnant subjects by Yü and associates (19). This

TABLE II

Clinical diagnosis and laboratory data from 28 prenatal patients *

Patient	Clinical diagnosis	Month of pregnancy	Serum creatinine	Serum uric acid	Arterial whole blood lactic acid	C_{urate}	C_{cr}	$C_{urate}:C_{cr}$
			mg/100 ml	*mg/100 ml*	*mg/100 ml*	*ml/min*	*ml/min*	%
M.W.	Normal	9	0.83	4.9	10.0	11.6	135	8.2
M.H.	Normal	7	0.71	3.9	9.0	11.7	150	7.8
D.B.	Normal	8	0.92	4.4	6.2	5.2	94	5.5
E.S.	Normal	9	0.78	3.7	6.3	7.5	80	9.4
A.M.	Normal	9	1.0	4.9	7.2	7.9	94	8.4
N.M.†	Normal	9	0.86	7.2	8.5	1.5	89	1.7
H.B.	Normal	7	0.74	2.9	5.8	12.3	112	11.0
S.S.	Normal	9	0.70	3.6	7.3	10.2	102	10.0
C.W.	Normal	9	0.69	4.1	7.1	9.9	120	8.3
V.B.	Normal	7	0.68	3.0	7.7	9.9	118	8.4
W.W.	Chronic hypertension	9	0.73	5.0	5.8	9.5	124	7.7
B.P.	Chronic hypertension	8	0.70	4.1	5.7	10.0	110	9.1
T.S.	Chronic hypertension	8	0.71	3.8	7.1	10.6	127	8.3
B.M.†	Chronic hypertension	7	0.71	3.9	18.0	5.7	132	4.3
G.Rea.	Pre-eclampsia	9	0.79	4.7	9.0	16.7	152	11.0
M.Sa.	Pre-eclampsia	9	0.64	3.1	4.9	16.0	160	10.0
M.St.	Pre-eclampsia	9	0.85	5.1	9.6	5.2	80	6.5
P.Z.	Pre-eclampsia	9	0.48	2.8	7.3	16.4	126	13.0
R.F.	Pre-eclampsia	9	0.77	5.7	13.0	5.5	86	6.4
R.T.	Pre-eclampsia	9	0.86	6.5	8.3	4.5	98	4.6
M.B.	Pre-eclampsia	8	0.97	6.7	11.0	1.0	63	1.6
D.M.	Pre-eclampsia	8	1.0	6.3	6.5	10.5	177	5.8
E.N.	Pre-eclampsia	7	0.6	4.0	5.3	6.5	59	11.0
G.Ree.	Pre-eclampsia	9	1.0	7.0	7.4	3.4	66	5.2
B.O.	Pre-eclampsia	7	1.4	10.0	11.0	3.2	92	3.5
D.A.†	Eclampsia	8	0.96	9.1	18.0	3.3	65	5.1
F.W.†	Eclampsia	9	1.4	11.0	16.0	1.0	57	1.8
L.L.	Eclampsia	9	0.74	6.8	12.0	1.7	77	2.3

* See footnote to Table I for abbreviations.
† Studied again in postpartum period.

effect of lactate has been recognized for many years, but the mechanism has not been completely clarified. The alteration in urate clearance after lactate infusion does not depend upon changes in glomerular filtration rate as measured by inulin (19) or creatinine clearance, and it is unlikely that lactate causes a selective change in the filtrability of serum urate. The most likely possibility is that lactate evokes greater net tubular reabsorption of uric acid. This could be due to increased reabsorption of filtered urate, diminished tubular urate secretion, or a combination of tubular reabsorptive and secretory changes.

A large body of evidence demonstrating reduced glomerular filtration rate in toxemia of late pregnancy has been accumulated, and there is no reason to doubt that reduction in the filtered urate load would lead to low uric acid clearance. However, the available data suggest that increased net tubular reabsorption of urate is common in toxemia (1, 2). On the other hand, examination of the reports emphasizing the role of glomerular changes reveals that serum uric acid concentrations were elevated far more than was the relative elevation of other metabolites whose excretion depends primarily upon the rate of glomerular filtration (20), and that uric acid clearances were depressed relatively more than creatinine clearances were depressed (21). The data of this study suggest that the reduction in urate clearance due to increased net tubular reabsorption is related to the effect of excessive lactic acid. Other metabolites may also contribute to the phenomenon.

Evidence in the literature suggests that the uterus and its contents are a major source of lactic acid in pre-eclampsia and eclampsia. A marked reduction in uterine and placental blood flow in toxemic patients has been demonstrated by several workers (22–24). These observations probably explain the low oxygen content of umbilical cord blood of infants at delivery of toxemic mothers (25–27). Hendricks (18) found a gradi-

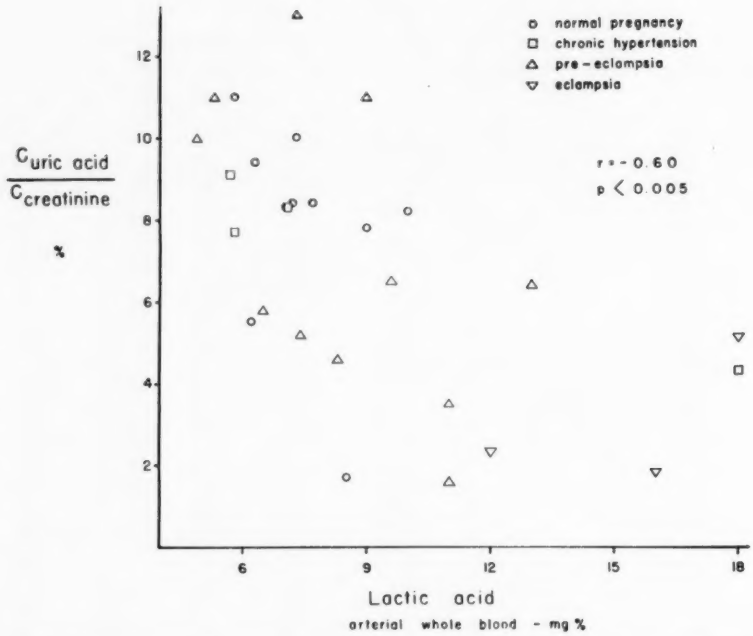

FIG. 2. RELATIONSHIP OF RESTING LACTIC ACID CONCENTRATION IN ARTERIAL BLOOD TO THE URIC ACID CLEARANCE: CREATININE CLEARANCE RATIO, IN NORMAL AND IN TOXEMIC PREGNANT PATIENTS. Data are shown from 10 normal, 11 pre-eclamptic, 3 eclamptic, and 4 chronic hypertensive pregnant patients. There is a statistically significant inverse relationship between the two parameters.

ent for lactic acid between normal fetus and normal mother at elective cesarian sections. Levels were highest in umbilical arterial blood and then in umbilical venous blood, maternal uterine venous blood and maternal peripheral venous blood, in descending order. The gradient was interpreted as evidence for net lactate transport from fetus to mother. However, Friedman and colleagues (28), working with normal pregnant rhesus monkeys at term, demonstrated bidirectional lactate exchange between fetal and maternal organisms. The authors felt that their results invalidated the concept of a unidirectional lactate transfer based on concentration gradients. The direction of net lactate

TABLE III

*Prenatal diagnosis and postnatal laboratory data from four subjects with low $C_{urate}:C_{cr}$ * in a prenatal study*

Patient	Prenatal clinical diagnosis	Serum creatinine	Serum uric acid	Arterial whole blood lactic acid	C_{urate}	C_{cr}	$C_{urate}:C_{cr}$
		mg/100 ml	*mg/100 ml*	*mg/100 ml*	*ml/min*	*ml/min*	%
N.M.	Normal	0.82	6.7	7.2	7.4	105	7.0
B.M.	Chronic hypertension	0.88	6.0	7.1	14.1	110	13.0
D.A.	Eclampsia	0.87	7.8	6.5	11.5	120	9.6
F.W.	Eclampsia	1.1	6.7	8.2	9.8	81	12.0

* See footnote to Table I for abbreviations.

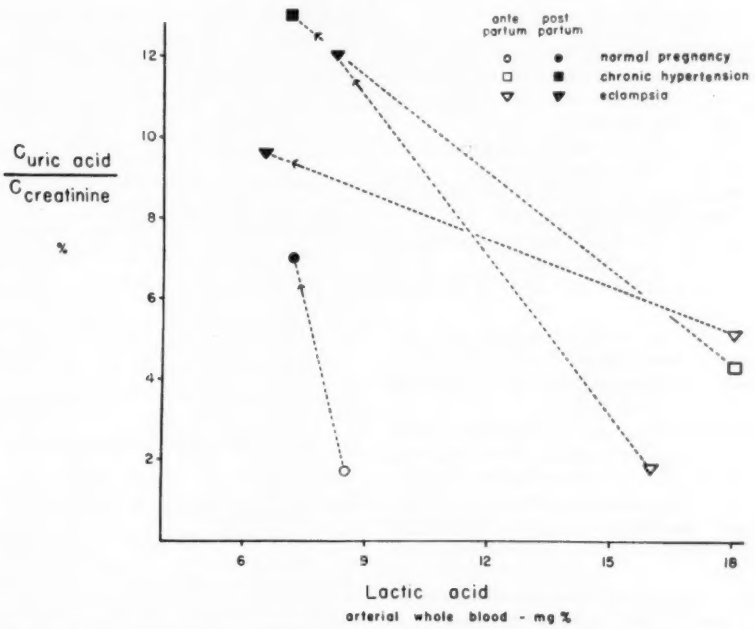

FIG. 3. RELATIONSHIP OF RESTING LACTIC ACID CONCENTRATION IN ARTERIAL BLOOD TO THE URIC ACID CLEARANCE: CREATININE CLEARANCE RATIO, BEFORE AND AFTER DELIVERY. The lactic acid level fell in three of four patients and the clearance ratio rose in all four patients, following delivery.

exchange and its quantitative characteristics are thus unresolved, but it would be reasonable to assume that the more hypoxic fetus of a pre-eclamptic patient, depending more upon anaerobic metabolism, produces greater than normal quantities of lactic acid which might then influence maternal-fetal exchange so that the rate of net lactate transfer into maternal blood is increased. Other tissues may also contribute to the lactate pool by increased production or diminished utilization. In any case, there is evidence that the concentration of lactic acid in the blood of toxemic patients is generally higher than that in normal pregnant subjects, and the data of this study suggest that the excessive lactate is at least partially responsible for the low urate clearance in these patients.

SUMMARY

The role of lactic acid in the low urate excretion in toxemia of pregnancy was studied. After obtaining the expected fall in the urate clearance of normal pregnant subjects by intravenous lactate infusion, determinations were made of endogenous arterial lactate concentrations and simultaneous urate and creatinine clearances in 28 (10 normal, 18 toxemic) subjects in late pregnancy.

There was a significant negative correlation between arterial whole blood lactic acid concentration and the ratio of uric acid clearance to endogenous creatinine clearance.

The correlation suggests that an increased level of lactic acid causes increased net renal tubular reabsorption of filtered urate and is responsible, at least in part, for the low uric acid clearance in toxemia of late pregnancy.

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URINARY OUTPUT OF ADRENALINE, NORADRENALINE, AND 3-METHOXY-4-HYDROXYMANDELIC ACID FOLLOWING CENTRIFUGATION AND ANTICIPATION OF CENTRIFUGATION *

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It has been shown that various forms of stress, i.e., thermal burn (1), muscular exercise (2), centrifugation (3–5), trauma (6) and so forth activate the sympatho-adrenal system and that this activation is manifested by an increase in the urinary output of adrenaline and noradrenaline. Furthermore, it has been demonstrated in the normal human that 3-methoxy-4-hydroxymandelic acid is one of the principal metabolic products of both adrenaline and noradrenaline (7–10). Hitherto, there have been no stress experiments correlating the urinary output of adrenaline and noradrenaline with that of 3-methoxy-4-hydroxymandelic acid. The experiments herein described not only demonstrate such a correlation but also show the effect of centrifugation and anticipation of centrifugation upon the urinary output of adrenaline, noradrenaline, and 3-methoxy-4-hydroxymandelic acid.

METHOD

A. Centrifugation

Nine healthy adult males were centrifuged on three different days to a 12 G forward acceleration to subjective tolerance. The ride did not exceed 3 minutes' duration and the rate of onset was 1 G per 5 seconds. During the time of centrifugation, the subjects in the free-swinging end of the WADD/human centrifuge were supported by a contoured net reclining seat (11). With this particular restraint system, subjects in a real centrifugation (12 G) faced the center of rotation with the head and spine forming an angle of 78° with the acceleration vector; the thighs and trunk forming an angle of 90° and the knees an angle of approximately 90°.

In order to evaluate the effect of anticipation upon the sympatho-adrenal system, some of the subjects were given mock centrifuge rides. The mock centrifugations were identical in all respects with those of a real centrifugation, except that subjects were spun at 2 rpm, equivalent to 0.02 G acceleration, for 3 minutes. However, to insure that no individual would be cognizant as to whether he was or was not to be centrifuged, the following random distribution of mock rides was instigated. The mock rides among the 9 subjects were such that 2 subjects had 3 real centrifugations, 2 subjects had 2 mock and 1 real centrifugation, and 5 subjects had 1 mock and 2 real accelerations, for a total of 18 actual and 9 mock centrifugations. As a further precaution to insure that the mock accelerations were unknown, subjects were not permitted to discuss their centrifuge rides.

B. Urine collections

During the 24 hours prior to experimentation, subjects were requested to refrain from ingesting coffee, tea, fruits, and all foods containing vanilla. This was done in order to avoid the accumulation of phenolic acids in the urine which might interfere with the detection of 3-methoxy-4-hydroxymandelic acid.

All subjects voided 2 hours before centrifugation and the urine was discarded. Thereafter, samples were collected approximately 1 hour before and 10 minutes before centrifugation; post-run samples were collected at approximately 30 minutes and 1 hour after centrifugation. Aliquots were preserved with a crystal of thymol and stored at $-10°$ C. In order to insure an adequate volume of urine, subjects drank 500 ml of water immediately after voiding the 1-hour pre-acceleration sample.

All control urine samples were obtained 3 weeks following experimentation. The urine was collected for 2 to 3 hours and at approximately the same time of day on which the subjects were originally centrifuged. All urine samples were assayed for adrenaline, noradrenaline, and 3-methoxy-4-hydroxymandelic acid (see paragraphs C and D, following).

C. Urinary adrenaline and noradrenaline

a) Preparation of urine extract. The procedure was adopted from von Euler and Hellner (12). The urine was hydrolyzed and the adrenaline and noradrenaline selectively adsorbed on aluminum hydroxide and filtered. The precipitate was washed and redissolved with 2 N sulphuric acid. The remaining salts were precipitated out by mixing the extract with alcohol and acetone. The filtrate was concentrated *in vacuo*.

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TABLE I

Average rate of urinary excretion of adrenaline in response to real and mock centrifugation in micrograms per hour

		Time in minutes				
	Control	−74	−11	0	+28	+89
Real centrifugation	0.86	1.03	1.38	Centrifugation	2.27	1.33
SD	±0.25	±0.09	±0.54		±1.02	±0.44
p*		<0.4	<0.01		<0.001	<0.01
Mock centrifugation	0.86	1.02	1.30		1.93	1.06
SD	±0.25	±0.61	±0.67		±0.77	±0.09
p*		<0.8	<0.05		<0.01	<0.2

* Comparison of experimental with control.

b) Bioassay of urine for adrenaline and noradrenaline. This procedure has been previously described (1, 12). In brief, the cat's blood pressure, which is sensitive to noradrenaline, is used in conjunction with the fowl's rectal cecum, which is sensitive to adrenaline. The cat's blood pressure was recorded from the carotid artery and injections of adrenaline, noradrenaline, and urinary extract were made into the femoral vein. The fowl's rectal cecum was suspended in a water bath containing Tyrode's solution at 39° C. Through the Tyrode's solution, 6.5 per cent of CO_2 in oxygen was bubbled. Injections of adrenaline, noradrenaline, and urinary extract were made into the bath and the degree of cecal relaxation was recorded on a kymograph.

c) Computation of results. Having determined the activity ratio for adrenaline and noradrenaline on the cat's blood pressure and on the hen's rectal cecum, and the activity of the unknown urinary extract in terms of L-noradrenaline, it is possible to calculate the relative amounts of adrenaline and noradrenaline in the urinary extract (1, 12).

D. Urinary 3-methoxy-4-hydroxymandelic acid

a) General. Urine samples, collected as outlined above, were extracted and the 3-methoxy-4-hydroxymandelic acid present was identified by two-dimensional paper partition chromatography. Quantitative estimation of 3-methoxy-4-hydroxymandelic acid was determined spectrophotometrically.

b) Preparation of urine extract. The method used was adopted from Armstrong (13) as modified by Berman and Pettitt (14). In brief, an aliquot of a measured volume of urine sample was acidified with 3 N HCl and extracted in ethyl acetate. After evaporation of the ethyl acetate, the residue was redissolved in absolute ethanol. The ethanol extract (0.5 ml) was applied to the filter paper with a specially designed semimicro automatic constant rate infusion pump and chromatographed in a two-phase solvent system. Three-methoxy-4-hydroxymandelic acid was visualized by spraying the chromatogram with a freshly prepared solution of diazotized *p*-nitroaniline.

c) Quantitative analysis of urinary 3-methoxy-4-hydroxymandelic acid. The details of this procedure were described by Berman and Pettitt (14). In brief, chromatographic areas containing the unknown were compared with known concentrations of 3-methoxy-4-hydroxymandelic acid. Areas containing unknown, known, and blanks of corresponding size were cut from the same chromatogram and eluted with an alcohol-sodium carbonate solution. The extinction coefficients for the eluates were determined against water at 520 mμ with a spectrophotometer. After subtraction of the blank values, a graph of the extinction coefficients against their concentration was made. The amounts of 3-methoxy-4-hydroxymandelic acid were calculated from this graph. Reproducibility averaged ± 5 per cent. Recoveries of added 3-methoxy-4-hydroxymandelic acid, 0.5 to 6 μg, were 95 ± 4 per cent.

Preformed urinary creatinine was determined by the method of van Pilsum, Martin, Kito and Hess (15).

RESULTS

Table I shows a comparison between the average urinary adrenaline output before and after centrifugation and before and after a mock centrifugation. From Table I, it is apparent that there is a 60 per cent increase in adrenaline output before actual centrifugation, and 51 per cent increase is seen before a mock centrifugation. There is a significant increase in adrenaline output after centrifugation as well as after a mock ride (Figure 1). It should be emphasized that the adrenaline output following centrifugation is not significantly different from that following mock centrifugation.

Table II shows a comparison between the urinary output of noradrenaline before and after centrifugation and before and after a mock centrifugation. From this table it is apparent that there is no significant increase in the noradrenaline output

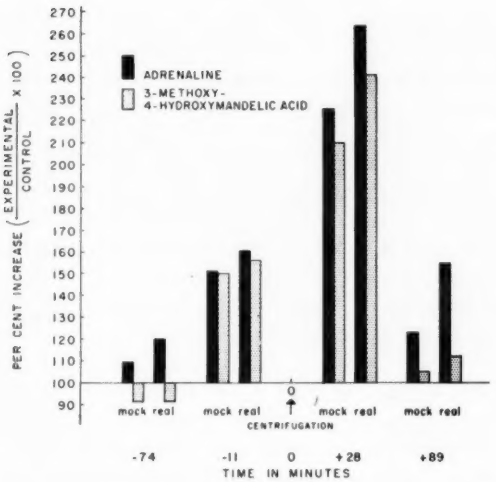

FIG. 1. AVERAGE PER CENT CHANGE OF ADRENALINE BEFORE AND AFTER CENTRIFUGATION AS COMPARED WITH THE PER CENT CHANGE IN 3-METHOXY-4-HYDROXYMANDELIC ACID. All figures reported in micrograms per hour.

immediately prior to both the true and the mock centrifugations. However, it should be noted that the greatest urinary output of noradrenaline follows actual centrifugation and is 53 per cent ($p < 0.05$) greater than the output following the mock centrifugation (Figure 2). Mock centrifugation per se produced an insignificant elevation in the output of noradrenaline.

Table III shows the average hourly rate of urinary excretion of 3-methoxy-4-hydroxymandelic acid before and after centrifugation and before and after mock centrifugation. The figures are the mean values for all subjects at the time of urine collections. From Table III it is evident that there was no significant difference in urinary excretion of 3-methoxy-4-hydroxymandelic acid in a real or mock acceleration. Further, there was a marked increase in the excretion of 3-methoxy-4-hydroxymandelic acid following actual centrifugation as well as following a mock centrifugation. This elevated 3-methoxy-4-hydroxymandelic acid output returned to control levels within 90 minutes after centrifugation.

As is shown in Figures 1 and 2, which illustrate the mean excretion rate of 3-methoxy-4-hydroxymandelic acid in each sampling period as a percentage change from control level, the increase was 56 per cent before a 12 G acceleration and 50 per cent prior to a mock centrifuge ride. In the period encompassing a real acceleration, there was an average increase of 141 per cent ($p < 0.001$). For mock accelerations, during this period, the increase was 110 per cent ($p < 0.01$).

Changes in creatinine excretion rate before and after real and mock acceleration were not statistically significant. Since the creatinine content of the urine has been used as the basis of determining the amount of 3-methoxy-4-hydroxymandelic acid present, the micrograms of 3-methoxy-4-hydroxymandelic acid per milligram of creatinine were calculated for each subject at the time of urine collection (Table III).

Figure 1 is a block graph showing a comparison between the adrenaline output and the 3-methoxy-4-hydroxymandelic acid output during similar periods before and after real and mock centrifugation. From this graph it is quite apparent that as the adrenaline release increases, the 3-methoxy-4-hydroxymandelic acid output also increases.

Figure 2 is a block graph showing a compari-

TABLE II

Average rate of urinary excretion of noradrenaline in response to real and mock centrifugation in micrograms per hour

		Time in minutes				
	Control	−74	−11	0	+28	+89
Real centrifugation	1.56	1.67	2.20	Centrifugation	2.45	2.22
SD	±0.71	±1.17	±0.28		±1.09	±1.45
p*		<0.9	<0.2		<0.05	<0.3
Mock centrifugation	1.56	1.44	1.73		1.61	1.26
SD	±0.71	±0.39	±0.82		±0.69	±0.59
p*		<0.8	<0.8		<0.9	<0.5

* Comparison of experimental with control.

TABLE III

Average rate of urinary excretion of 3-methoxy-4-hydroxymandelic acid in response to real and mock centrifugation

	Control	Time in minutes −74	−11	0	+28	+89
Real centrifugation						
Mean 3-methoxy-4-hydroxymandelic acid excretion (μg/hr)	107.4	101.3	168.6		259.2	120.9
SD	±30.79	±29.20	±90.66		±97.84	±19.78
p*		<0.7	<0.1		<0.001	<0.8
Mean 3-methoxy-4-hydroxymandelic acid excretion (μg/mg creatinine)	1.5	1.7	2.4	Centrifugation	3.2	1.6
Mock centrifugation						
Mean 3-methoxy-4-hydroxymandelic acid excretion (μg/hr)	107.4	102.5	160.7		226.0	112.9
SD	±30.79	±30.83	±105.70		±83.50	±42.60
p*		<0.8	<0.2		<0.01	<0.8
Mean 3-methoxy-4-hydroxymandelic acid excretion (μg/mg creatinine)	1.5	1.7	2.3		3.2	1.6

* Comparison of experimental with control.

son between the noradrenaline output and the 3-methoxy-4-hydroxymandelic acid during similar periods before and after real and mock centrifugation. From Graph *2* it seems evident that, as the noradrenaline output rises, there is a concomitant rise in the 3-methoxy-4-hydroxymandelic acid output. However, this correlation of noradrenaline with 3-methoxy-4-hydroxymandelic acid is not so clearly delineated as it is with adrenaline.

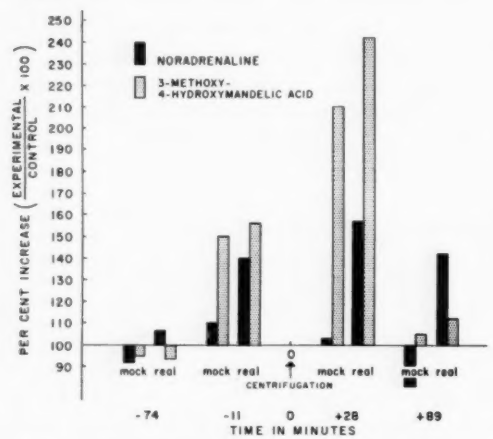

FIG. 2. AVERAGE PER CENT CHANGE OF NORADRENALINE BEFORE AND AFTER CENTRIFUGATION AS COMPARED WITH THE PER CENT CHANGE IN 3-METHOXY-4-HYDROXYMANDELIC ACID. All figures reported in micrograms per hour.

DISCUSSION

Of the adrenaline and noradrenaline normally released by the adrenal medulla and sympathetic nerves, only 3 to 4 per cent is excreted in the urine in the free and conjugated form (8, 9, 16, 17). The remainder of the released adrenaline and noradrenaline is rapidly metabolized and of the metabolic products, 3-methoxy-4-hydroxymandelic acid represents approximately *27* per cent of the metabolized adrenaline and *32* per cent of the metabolized noradrenaline (8, 9). Therefore, in view of 3-methoxy-4-hydroxymandelic acid's common origin, it is quite natural that any stressful situation which would activate the sympathetic nervous system into releasing noradrenaline and the adrenal medulla into releasing adrenaline would also produce an increase in the urinary 3-methoxy-4-hydroxymandelic acid. The experiments herein described illustrate just such a relation; i.e., with an increase in noradrenaline or an increase in adrenaline output, there is a commensurate rise in the 3-methoxy-4-hydroxymandelic acid excretion. Although 3-methoxy-4-hydroxymandelic acid is an excellent indicator of the sympatho-adrenal activity, it does not tell precisely what part of the sympatho-adrenal system is involved. In contrast, urinary adrenaline and noradrenaline do afford more precise information, since adrenaline is derived from the adrenal me-

dulla (18–20) and noradrenaline chiefly from the sympathetic nerves (20–23).

From Table I and Figure 1 it appears that the adrenaline release is closely geared to the emotions. This is evidenced by the markedly increased adrenaline release during the mock ride, that is to say, in anticipation of being centrifuged. Further, the adrenaline output prior to actual centrifugation and prior to a mock ride are similarly elevated; this would indicate that the anxiety associated with being centrifuged or anticipation of being centrifuged, as seen in the mock ride, is the chief factor concerned with the release of adrenaline. Certainly, the physical changes produced by actual centrifugation must cause some release of adrenaline but, at least in these experiments, these physical changes when compared with the emotional effect perform a relatively minor role in the release of adrenaline.

Noradrenaline is the neurohormone of the sympathetic nerves (21, 23, 24) and is of fundamental importance in the control of the vascular bed (24–26). Therefore one would expect that, under the stress of centrifugation, this hormone should be released in increased quantities and in amounts related to the extent of the stress. Table II and Figure 2 show that, following centrifugation, there is a 53 per cent ($p < 0.05$) increase in noradrenaline output. However, in contrast to actual centrifugation, mock acceleration showed little change (3 per cent) in the urinary output of noradrenaline. In general, the pre-centrifugation and pre-mock centrifugation outputs of noradrenaline were not significantly above the control values for noradrenaline (Table II). From these findings and those of other investigators (3), one would conclude that the physical stress of centrifugation activates the sympathetic nervous system and that this is manifested by an increase in the urinary output of noradrenaline. However, it should be pointed out that in these particular experiments, the physical stress of forward acceleration was for only 3 minutes and therefore, minimally activated the sympathetic nervous system, as was reflected by a slight increase in the noradrenaline output. The emotions or anticipation of being centrifuged appear to have little or no effect upon the release of noradrenaline. This is evidenced by the slight but insignifiant increase in noradrenaline release prior to and following a mock centrifugation (Table II and Figure 2).

This elevation of adrenaline associated with the anxiety of anticipating centrifugation and the elevation of noradrenaline following actual centrifugation emphasize the possible importance of these two hormones in various mental and physical stresses. Furthermore, the previous report of Goodall (19) that the adrenals of "fright and flight" animals, as do those of man (18, 24) contain predominantly adrenaline, becomes more meaningful when extrapolated in terms of anxiety and the preparation for flight; the adrenals of aggressive animals such as the lion contain predominantly noradrenaline. Adrenaline elevates the metabolism (24, 27, 28), increases the cardiac rate (29–31) and is a vasodilator (24, 32) in the muscles commonly involved in running; noradrenaline, on the other hand, is a potent vasoconstrictor (24, 32) with limited metabolic action (24, 28, 33, 34).

SUMMARY

The purpose of these experiments was to determine the effect of centrifugation (gravitational stress) and the anticipation of being centrifuged upon the release of adrenaline, noradrenaline, and their common metabolic product, 3-methoxy-4-hydroxymandelic acid. Nine Air Force subjects were centrifuged at 1 G per 5 seconds to 12 G or were given a mock ride. Each subject was unaware as to whether he would receive a real ride or a mock ride. It was therefore possible to measure the sympatho-adrenal response to both centrifugation and anticipation of centrifugation. Urine samples were collected before and after each ride and bioassayed for adrenaline and noradrenaline; 3-methoxy-4-hydroxymandelic acid was determined chromatographically.

Under high gravitational stress, increased adrenaline release seems to be largely related to the emotions, while the noradrenaline release seems more closely related to the physical changes (hemodynamics and so forth) produced by centrifugation. Following the increased release of either or both adrenaline and noradrenaline, there is a commensurate rise in the urinary output of their common metabolic product, 3-methoxy-4-hydroxymandelic acid.

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PLASMA ANTIDIURETIC ACTIVITY AND FREE WATER CLEARANCE FOLLOWING OSMORECEPTOR AND NEUROHYPOPHYSEAL STIMULATION IN HUMAN SUBJECTS

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Since the description of antidiuretic hormone (1) and the factors which influence its release from the neurohypophysis (*2*), renal investigators have been interested in correlating physiological and clinical situations to the plasma levels of this hormone. However, the accurate measurement of antidiuretic activity in human plasma has proved difficult because of the lack of a sensitive method of bioassay and the extremely low concentrations of antidiuretic hormone (ADH) which are present in peripheral blood.

Further interest in measuring antidiuretic activity in human plasma was prompted by two factors: *1*) the recent development of an improved method of bioassay of antidiuretic hormone in mammalian plasma (3, 4), and *2*) an opportunity to obtain blood from the superior vena cava where dilution and inactivation of antidiuretic hormone were considered to be minimal.

The purpose of this investigation was to employ procedures calculated to stimulate the secretion of ADH in normal human subjects and to correlate plasma levels of antidiuretic activity with free water clearance. Three patients with diabetes insipidus were also studied.

METHODS

The assay method for plasma antidiuretic activity has been previously described (4) and utilizes rats anesthetized with ethanol, under constant water load; a minor modification which has been introduced involves the injection into each assay animal of fixed amounts of standard vasopressin solutions, the antidiuretic response (instead of the dose of standard vasopressin solution administered) now being related to the rat's body weight. Plasma samples and standard solutions of aqueous vasopressin (Pitressin, Parke, Davis & Co., Lot no. 3-161-10W 107GA) were administered intravenously and in each instance the volume of plasma or standard injected was 0.7 ml; 0.1 ml of heparin saline solution (1:100) was used to wash in the test material. In all cases the human plasma was injected into the rat within 12 minutes after blood withdrawal. The same rats were used for measurement of plasma antidiuretic activity and standard vasopressin solutions except in rare instances in which an animal developed hematuria or oliguria.

The rats' urine was collected at 10-minute intervals and the degree of antidiuresis was expressed as an antidiuretic index per 100 g of rat body weight; i.e., the percentage change in urine specific gravity calculated from the average of two 10-minute samples preceding injection and the average of two 10-minute samples immediately following injection. The antidiuretic activity of the plasma samples was determined by relating the antidiuretic index obtained with plasma to the indices obtained with known concentration of vasopressin. The reported individual values of plasma antidiuretic activity are an average of the antidiuretic response obtained from all assay animals receiving an injection of the particular plasma sample.

It was recognized that measurement of the rats' free water clearance would offer a more accurate assessment of antidiuretic activity than would changes in urine density. However, such measurements were found to be impractical because of considerable lowering of plasma and urine freezing points produced by the alcohol used for anesthesia. Although the material responsible for the antidiuretic activity of human plasma is not conclusively known, the previous observations of Macfarlane and Robinson (5) suggest that it is possibly antidiuretic hormone. Using a similar bioassay procedure in rats, these workers compared the antidiuretic activity of human plasma with standard vasopressin solutions: parallel dose response curves and almost identical time flow curves were obtained, and it was also found that the antidiuretic activity of both the plasma and the vasopressin solutions could be inactivated by thioglycollic acid.

The 6 normal subjects were volunteer male medical students, and of the 3 patients, 2 had diabetes insipidus due to intracranial lesions and a third had renal diabetes insipidus resulting from gross bilateral hydro-

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TABLE I

Renal response and plasma antidiuretic activity following osmoreceptor and neurohypophyseal stimulation in normal subjects

Subject Age Height Weight	Time	Urine		Plasma (sup. vena cava)		Clearances	
		Flow	Osmolality	Osmolality	Antidiuretic activity	C_{osm}	C_{H_2O}
	min	*ml/min*	*mOsm/kg*	*mOsm/kg*	*μU equiv. vasopressin/ml*	*ml/min*	
R.N.				Fluid deprivation, 13 hrs			
23 yrs	−205–0	0.47	1,134	283	<30*	1.88	− 1.41
177 cm							
74 kg	0–80			Hydration: 2.5 L H_2O orally			
	80–105	11.2	49	284	<30†	1.93	9.27
	125–145	18.3	50			3.21	15.09
	145–148			Infuse 3 mg nicotine bitartrate, 1 mg/min			
	145–165	24.0	53	286	<30‡	4.45	19.55
	165–185	19.0	51			3.39	15.61
	205–225	15.5	50			2.71	12.79
	264			288			
	265–275			Infuse 450 ml 20% mannitol, 45 ml/min			
	265–285	20.8	388	293	110‡	27.54	− 6.74
	285–305	15.4	401			21.29	− 5.89
	305–325	11.5	500			19.96	− 8.46
W.W.				Fluid deprivation, 14 hrs			
24 yrs	−178–0	0.70	797	284	<30‡	1.96	− 1.26
185 cm							
86 kg	0–40			Hydration: 2.0 L H_2O orally			
	60–80	15.8	48	275	<30‡	2.76	13.04
	110–113			Infuse 3 mg nicotine bitartrate, 1 mg/min			
	100–120	13.8	58	285	<30‡	2.81	10.99
	120–160	3.0	310			3.26	− 0.26
	160–180	12.3	100			4.32	7.98
	180–200	17.8	51			3.19	14.61
R.R.				Fluid deprivation, 14 hrs			
23 yrs	−185–0	0.40	1,319	288	<30†	1.83	− 1.43
188 cm							
82 kg	0–70			Hydration: 2.5 L H_2O orally			
	80–100	9.2	76	278	<30‡	2.51	6.69
	140–160	10.2	60			2.20	8.00
	180–183			Infuse 3 mg nicotine bitartrate, 1 mg/min			
	160–190	12.7	55	285	<30*	2.45	10.25
	190–205	4.6	201			3.24	1.36
	205–225	8.0	81			2.27	5.73
	225–245	13.3	57	286		2.65	10.65
	250–260			Infuse 350 ml 20% mannitol, 35 ml/min			
	245–273	13.6	207	299	<30*	9.41	4.19
	273–297	8.4	415			11.74	− 3.34
D.F.				Fluid deprivation, 13 hrs			
23 yrs	−120–0	0.43	949	287	<30‡	1.42	− 0.99
178 cm							
76 kg	0–45			Hydration: 2.0 L H_2O orally			
	60–80	11.9	62	275	<30‡	2.68	9.22
	100–120	12.0	61			2.66	9.34
	132–135			Infuse 3 mg nicotine bitartrate, 1 mg/min			
	120–140	11.5	64	286	>200*	2.57	8.93
	140–160	1.2	533			2.24	− 1.04
	160–180	5.5	173			3.33	2.17
	180–200	14.2	67			3.33	10.87
	249			287	<30‡		
	250–262			Infuse 240 ml 20% mannitol, 20 ml/min			
	240–260	18.5	103			6.44	12.06
	260–280	6.9	447	296	<30*	10.42	− 3.52
	280–300	5.0	525			8.99	− 3.99

* Average of response in three rats.
† Average of response in four rats.
‡ Average of response in two rats.
§ Subject unable to void between first morning urine specimen (−210 min) and 100 minutes after oral administration of water; this second urine sample (320 ml) was discarded.

TABLE I—(*Continued*)

Subject Age Height Weight	Time	Urine		Plasma (sup. vena cava)		Clearances	
		Flow	Osmolality	Osmolality	Antidiuretic activity	C_{osm}	C_{H_2O}
	min	*ml/min*	*mOsm/kg*	*mOsm/kg*	*μU equiv. vasopressin/ml*	*ml/min*	
D.T.			Fluid deprivation, 14 hrs				
22 yrs	−210		1,040				
170 cm							
68 kg	0	§		287	40.0*		
	0–60		Hydration: 3.5 L H_2O orally				
	90			277	<30‡		
	100–120	18.3	117			7.73	10.57
	120–140	14.3	52			2.69	11.61
	143–146	Infuse 3 mg nicotine bitartrate, 1 mg/min					
	140–160	15.7	49	282	>200*	2.73	12.97
	160–180	14.5	53			2.72	11.78
	200–220	16.6	76			4.43	12.17
	240–260	22.6	73			5.77	16.83
	265			286			
	266–276	Infuse 400 ml 20% mannitol, 40 ml/min					
	260–280	27.1	149			13.60	13.50
	280–300	19.2	233	297	<30*	15.06	4.14
	300–320	24.7	166			14.14	10.56
G.H.			Fluid deprivation, 14 hrs				
24 yrs	−195–0	0.46	1,115	288	<30‡	1.78	− 1.32
175 cm							
67 kg	0–70		Hydration: 3.0 L H_2O orally				
	80–100	10.5	87	275	<30*	3.32	7.18
	140–160	12.7	62			2.86	9.84
	167–170	Infuse 3 mg nicotine bitartrate, 1 mg/min					
	173–175	Infuse 2 mg nicotine bitartrate, 1 mg/min					
	160–180	16.7	60			3.58	13.12
	180–200	17.8	64	282	<30*	4.04	13.76
	220–240	20.9	61	286		4.46	16.44
	250–270	Infuse 250 ml 20% mannitol, 12.5 ml/min					
	240–265	18.1	110			6.73	11.37
	265–280	10.4	445	296	<30*	15.64	− 5.24
	280–300	6.2	434			9.28	− 3.08

nephrosis. The superior vena cava (SVC) was catheterized using a no. 6 Cournand cardiac catheter, the position of the catheter tip being checked fluoroscopically. Because of the possibility of anomalous venous drainage of the posterior pituitary gland, it was decided to bypass the internal jugular veins for the certainty of obtaining blood from the neurohypophysis. Blood samples for the determination of plasma osmolality and antidiuretic activity were taken from the SVC. Throughout each experiment urine collections were obtained at frequent intervals (usually 20 minutes) by requesting the subjects to void normally. In the patients with diabetes insipidus urine samples were obtained by means of an indwelling bladder catheter.

Plasma and urine osmolality were determined using a Fiske osmometer. Osmolal clearance and free water clearance were calculated as follows. Osmolal clearance in milliliters per minute, $C_{osm} = U_{osm}V/P_{osm}$ (U_{osm} = urine osmolality, mOsm per kg; P_{osm} = plasma osmolality, mOsm per kg; V = urine flow, milliliters per minute); free water clearance in milliliters per minute, $C_{H_2O} = V - C_{osm}$.

The subjects and patients refrained from food and liquid for at least 12 hours prior to reporting for the experiment. The degree of dehydration was assessed by determining the loss in body weight from the evening before to the morning of the experiment.

The first blood sample was taken after a period of 13 to 14 hours' dehydration; following this the subject or patient was encouraged to drink from 2 to 3 L of water and the second blood sample was withdrawn during water diuresis. After the initial water loading and until the end of the experiment, a constant degree of hydration was maintained by the oral administration of water or orange juice in quantities equivalent to the urine volume.

Approximately 1 hour following the removal of the second blood sample, nicotine bitartrate (3 to 5 mg) was infused into a peripheral vein and 5 to 10 minutes later the third blood sample was obtained. The last blood sample for bioassay was withdrawn 5 to 10 minutes after 240 to 450 ml of 20 per cent mannitol had been infused into a peripheral vein.

In the two patients with diabetes insipidus due to intracranial lesions, an intravenous infusion of vasopressin (50 mU per hour) was given at the end of the investigation.

RESULTS

Normal subjects. The results are listed in Table I. The weight lost during the 13 to 14 hour dehydration period varied from 1 to 1.5 kg. In five of the six subjects the plasma levels of antidiuretic activity in the dehydrated state were too low to demonstrate any difference from those in the hydrated state. At the end of the dehydration period, the free water clearance had a negative value (-0.99 to -1.41 ml per minute), while clearances of up to 13 ml per minute were recorded at the time of blood withdrawal in the hydrated state. In one subject (D.T.) in whom a measure of free water clearance was not obtained during dehydration, the ratio of urine to plasma osmolality was 3.62. This latter subject, who was unable to void at the end of the dehydration period, had the greatest weight loss during fluid deprivation, and was the only subject in whom plasma antidiuretic activity could be demonstrated (40 μU equivalents per ml) during the dehydrated state.

The response to the intravenous infusion of nicotine was variable. Two of the six subjects (R.N. and G. H.) had no side effects after nicotine bitartrate (3 to 5 mg) and there was no antidiuretic response nor detectable increase in plasma antidiuretic activity. W.W. and R.R. both experienced side effects following 3 mg of nicotine bitartrate, consisting of paresthesia, slight nausea and dizziness and exhibited a marked decrease in urine flow and free water clearance; however, a significant change in plasma antidiuretic activity could not be detected in either subject. D.F., who also experienced paresthesia and dizziness, had an antidiuresis associated with a large increase in plasma antidiuretic activity. A similar increase in plasma activity occurring in D.T. was associated with similar side effects but there was only a minimal reduction in urine flow and free water clearance.

Following the infusion of 20 per cent mannitol a marked decrease in free water clearance was observed in all subjects, but in only one (R.N.) was there a demonstrable increase in plasma antidiuretic activity.

Patients with diabetes insipidus. The results are shown in Table II. The weight lost during the 12 to 13.5 hours of dehydration was considerably greater than in the normal subjects, being 2.3 kg in M.J., 2.1 kg in W.K. and 4.6 kg in J.N. The patient with renal diabetes insipidus (W.K.) had high levels of plasma antidiuretic activity which disappeared following hydration, whereas in the two patients with disease of the pituitary-hypothalamic type (M.J. and J.N.), values of less than 30 μU equivalents of vasopressin per ml plasma were obtained in both the dehydrated and hydrated states. After 12 hours' dehydration in M.J., the urine osmolality rose slightly above that of the plasma, giving a free water clearance of -0.14 ml per minute. Simultaneous creatinine clearances were measured in this case; these showed no significant difference between the periods of dehydration and hydration, being 85 and 82 ml per minute, respectively (the cause of the slight impairment of renal function was not ascertained). The administration of nicotine in M.J. caused mild side effects and, although followed by a demonstrable increase in plasma antidiuretic activity, there was no significant decrease in free water clearance. Vasopressin administration in M.J. and J.N. produced negative values for free water clearance; this did not occur in the patient with renal diabetes insipidus (further details of this case including the results of vasopressin infusions will be published in a separate report).

DISCUSSION

The very low levels of plasma antidiuretic activity in normal subjects at the end of a 13 to 14 hour dehydration period agree well with the values previously reported by Bisset (6) and Heller (7) on internal jugular venous blood from human subjects. In nine patients, at surgical operation, Bisset recorded a detectable antidiuretic activity in only two cases, the values being 12 and 20 μU per ml blood, while in three subjects (not under general anesthesia), Heller obtained values of less than 20 μU per ml in two and less than 11 μU per ml in the third subject.

There remains little doubt that levels of plasma antidiuretic activity of less than 30 μU equivalents of vasopressin per ml are capable of producing the degree of antidiuresis obtained in our dehydrated subjects. Theobald (8) has demonstrated that a single intravenous injection of 5 to 10 mU of vasopressin will produce almost maximal antidiu-

TABLE II

Renal response and plasma antidiuretic activity following osmoreceptor and neurohypophyseal stimulation in patients with diabetes insipidus

Patient Diagnosis Age, Sex Height Weight	Time	Urine		Plasma (sup. vena cava)		Clearances	
		Flow	Osmolality	Osmolality	Antidiuretic activity	C_{osm}	C_{H_2O}
	min	*ml/min*	*mOsm/kg*	*mOsm/kg*	*μU equiv. vasopressin/ml*	*ml/min*	
M.J.			Fluid deprivation, 12 hrs				
Diabetes insipidus following hemorrhage from eosinophilic adenoma of pituitary. 42 yrs, ♀ 160 cm 63 kg	−30–0	1.7	339	313	<30*	1.84	− 0.14
	0–75		Hydration: 2.5 L H_2O orally				
	75–90	4.9	170			2.80	2.10
	90–105	4.7	160	295	<30†	2.55	2.15
	105–120	5.2	164			2.90	2.30
	161–164		Infuse 3 mg nicotine bitartrate, 1 mg/min				
	165–180	5.7	166	292	41.0†	3.24	2.46
	180–195	5.5	173			3.26	2.24
	210–225	5.1	185			3.23	1.87
	225–285		Infuse vasopressin 50 mU/hr				
	225–240	2.8	193			1.86	0.94
	240–255	2.5	300			2.58	− 0.08
	255–270	1.3	420	291		1.88	− 0.58
	270–285	2.0	513			3.53	− 1.53
W.K.			Fluid deprivation, 12 hrs				
Renal diabetes insipidus due to hydro-nephrosis 29 yrs, ♂ 161 cm 55 kg	−25–0	2.5	134	301	>200†	1.11	1.39
	0–75		Hydration: 2.0 L H_2O orally				
	75–90	4.7	122			1.96	2.74
	90–105	5.7	110	291	<30†	2.14	3.56
	105–120	5.3	110			2.00	3.30
J.N.			Fluid deprivation, 13.5 hrs				
Diabetes insipidus following head injury 16 yrs, ♂ 183 cm 99 kg	−160–0	2.7	236	298	<30*	2.14	0.56
	0–95		Hydration: 3.5 L H_2O orally				
	100–120	14.3	58	294	<30*	2.82	11.48
	160–180	11.8	58			2.37	9.43
	215			288			
	236–239		Infuse 3 mg nicotine bitartrate, 1 mg/min				
	220–240	12.0	59			2.48	9.52
	240–265	11.1	62	281	<30*	2.45	8.65
	265–290	10.6	63			2.38	8.22
	295–305		Infuse 400 ml 20% mannitol, 40 ml/min				
	305–380		Infuse 450 ml 20% mannitol, 6 ml/min				
	290–320	28.8	165	302	<30‡	15.74	13.06
	320–340	31.5	200			20.93	10.57
	340–360	31.5	205	300		21.52	9.98
	360–380	29.5	209			20.55	8.95
	380–440		Infuse vasopressin 50 mU/hr and 180 ml 20% mannitol, 3 ml/min				
	380–400	25.0	242			20.37	4.63
	400–420	17.0	291	295		16.77	0.23
	420–440	16.2	296			16.31	− 0.11

* Average response in three rats.
† Average response in two rats.
‡ Average response in five rats.

resis in man; assuming a distribution of the hormone which is limited to the plasma volume, such doses would result in plasma concentrations of approximately 2 to 4 μU per ml. Further work in human subjects (9–11) has shown that maximal antidiuresis can be achieved by the intravenous administration at rates of 50 mU or less per hour.

The individual variation encountered after nicotine administration in human subjects is well documented (12–14) and receives further confirmation from the results of this study. R.N. (nonsmoker) and G.H. (smoker) had no side effects, decrease in C_{H_2O} or increase in plasma antidiuretic levels following nicotine infusion. This lack of response is consistent with the view that these two subjects were insensitive to the dose of nicotine administered, and no appreciable quantities of ADH were liberated from the neurohypophysis. Both W.W. and R.R. had significant side effects and an antidiuretic response, but there was no detectable increase in plasma antidiuretic activity. While there is little doubt that ADH release was stimulated in both of these subjects, the concentrations in the superior vena caval blood at the time of sampling were too low to permit detection. D.F. showed a marked increase in plasma antidiuretic activity which was associated with a renal response. The other normal subject showing a well marked increase in plasma antidiuretic activity (D.T.) experienced paresthesia and lightheadedness but there was only a minimal reduction in free water clearance. A similar anomalous result following nicotine occurred in one of the patients with diabetes insipidus (M.J.). In these two instances the possibility that the plasma produced antidiuresis in the rats by means of a non-ADH mechanism cannot be entirely excluded since glomerular filtration and solute excretion rates were not measured in these animals. The possibility that the nicotine infused into the human subject had any direct effect upon the liberation of ADH in the rats used for assay can be excluded with reasonable certainty. Burn, Truelove and Burn (13) have demonstrated that extremely large doses of nicotine must be injected into a rat before any antidiuretic effect is noted. This dose would have to be at least 1,000 times greater than the amount of nicotine the rats actually received in our assays.

The intravenous infusion of 20 per cent mannitol was associated in each normal subject with a rise in plasma osmolality and a pronounced decrease in free water clearance. The latter finding was attributed to ADH release from osmoreceptor stimulation and did not occur in J.N., the patient with diabetes insipidus, in whom a mannitol infusion was performed. Of the two subjects (R.N. and G.H.) showing the greatest reduction in C_{H_2O}, only R.N. had a detectable increase in plasma antidiuretic activity. However, due to infusion difficulties in G.H., the mannitol was delivered over a 20 minute period instead of the usual 10 minutes. This slow rate of mannitol infusion may have initiated a slow continuous stimulation of the osmoreceptors and thus a steady but small release of ADH which was not detectable by the assay method. In fact, this type of slow continuous release may indeed be the reason for our inability to detect any appreciable change in plasma antidiuretic levels following the mannitol infusion in any of the other subjects. The reports of Theobald (8) and others (9–11) demonstrate that very small quantities of vasopressin which could not be measured by our assay method may produce the degree of renal response noted.

The bioassay method used in this investigation for the measurement of plasma antidiuretic activity has several advantages over those previously described in that it utilizes animals with their endogenous ADH secretion inhibited by ethanol, a constant water load, intravenous injections of plasma samples into rats within 12 minutes of blood withdrawal from the superior vena cava, and the very sensitive criterion of potency of change in urine specific gravity. However, it is evident from the results obtained that small amounts of antidiuretic hormone (less than 30 μU equivalents of vasopressin per ml of plasma), which are sufficient to produce a marked renal response, cannot be readily detected. We consider that further progress in the measurement of antidiuretic hormone in plasma is now dependent upon the development of a satisfactory method of concentrating the hormone before injection into the assay animal.

SUMMARY

Plasma antidiuretic activity has been measured in six healthy male subjects, using an improved rat bioassay method which included intravenous in-

jections and change in urine specific gravity as the sensitive criterion of potency. Studies were also made in three patients with diabetes insipidus, one of whom had nephrogenic disease. Blood for assay was withdrawn from the superior vena cava to minimize dilution and inactivation of antidiuretic hormone. Observations were made during dehydration and water diuresis and following intravenous infusions of nicotine and mannitol. The plasma levels of antidiuretic activity were compared with changes in free water clearance.

In five of the six normal subjects the plasma levels of antidiuretic activity in the dehydrated state were too low (30 μU equivalents of vasopressin per ml) to allow differentiation from those obtained after water loading. One patient with renal diabetes insipidus was shown to have high levels of plasma antidiuretic activity which disappeared following hydration, whereas two other patients, with intracranial lesions, had less than 30 μU equivalents of vasopressin per ml of plasma in both the dehydrated and hydrated states.

The response to nicotine was extremely variable and ranged from high levels of antidiuretic activity and negative values of free water clearance to no detectable response in either of these parameters. The infusion of mannitol in the normal subjects resulted in a pronounced decrease in free water clearance, but in only one instance was there a demonstrable increase in plasma antidiuretic activity.

Despite the use of optimal methods, employing a sensitive bioassay technique in conjunction with blood sampling from the superior vena cava, the concentrations of antidiuretic hormone in plasma are two low in the majority of physiological and clinical situations to be accurately measured. It is suggested that further progress in the measurement of antidiuretic hormone in human plasma lies in the development of a satisfactory method of concentrating the hormone before injection into the assay animal.

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CEREBROSIDE SYNTHESIS IN GAUCHER'S DISEASE *

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Familial lipodystrophic conditions, such as Gaucher's, Niemann-Pick, and Tay-Sachs disease, are characterized by the intracellular accumulation of abnormally large quantities of sphingolipids. In Gaucher's disease, the offending lipids are cerebrosides, while Niemann-Pick and Tay-Sachs diseases are characterized by the accumulation of sphingomyelin and gangliosides, respectively. In Gaucher's and Niemann-Pick disease, involvement of the reticuloendothelial system is extensive, and splenomegaly and hepatomegaly are frequently observed. Lieb in 1924 (1) identified the lipid stored in reticuloendothelial cells in a case of Gaucher's disease as a cerebroside. His findings were supported by other investigators (2, 3), and eventually Halliday, Deuel, Tragerman and Ward (4) and Rosenberg and Chargaff (5) presented evidence that the accumulated cerebrosides in Gaucher's disease contained predominantly glucose instead of the high content of galactose usually present in cerebrosides.

It was felt that techniques developed in this laboratory for studies of the biosynthesis of sphingosine and cerebrosides might be useful for investigations of cerebroside formation in tissue samples obtained from patients with Gaucher's disease. The data obtained by Brady and Koval (6), Burton, Sodd and Brady (7), and Cleland and Kennedy (8) indicate that cerebroside synthesis probably occurs in the following fashion: palmitic aldehyde + serine → sphingosine + CO_2; sphingosine + uridinediphosphate galactose → psychosine + uridinediphosphate; psychosine + fatty acid → cerebroside. Since purified enzyme systems which catalyze the biosynthesis of cerebrosides were not available, tissue slices were employed in the present investigations to study the formation of these sphingolipids. Tissue was occasionally available from patients who were splenectomized to arrest the pancytopenia of hypersplenism. It was not possible to obtain control specimens of splenic tissue suitable for metabolic studies from normal children of the same age as the patients with Gaucher's disease. Therefore, the results obtained in spleens from Gaucher's patients were compared with those obtained in two children with Niemann-Pick disease and in one adult case of idiopathic thrombocytopenic purpura.

Experiments were designed to explore several factors that might be thought to contribute to the pathogenesis of Gaucher's disease. Enzymic disturbances conceivably could lead to an alteration of normal processes, causing a replacement of a large proportion of cerebroside galactose by glucose. The metabolism of such an "abnormal" cerebroside might be imagined to proceed at a slower rate than the characteristic galacto-cerebroside. Also, attention was directed to the rate of formation of the total cerebroside molecule compared with the rate observed in tissue samples obtained from patients who did not show signs of cerebroside accumulation.

EXPERIMENTAL METHODS

The spleens used in this study were refrigerated following splenectomy and processed within 15 to 30 minutes. Slices measuring $10 \times 10 \times 0.5$ mm were prepared in the cold and incubated aerobically in Krebs-Ringer bicarbonate solution (9), pH 7.5, for 3 hours at 37° C. The following substrates were added to the incubation mixtures in amounts ranging from 0.5 to 5.0 μmoles per flask: uniformly-labeled glucose-C^{14} (0.89 μc per μmole); galactose-1-C^{14} (0.44 μc per μmole); and sodium acetate-1-C^{14} (5.8 μc per μmole). Each substrate was incubated with the tissue slices in triplicate. Non-incubated samples also in triplicate were used as controls for determining the reliability of the isolation and recovery techniques.

After incubation, the cerebrosides were isolated essentially according to the method described by Radin, Brown and Lavin (10). The contents of the incubation flasks were homogenized with an equal volume of 10 per cent ice-cold trichloroacetic acid and centrifuged. The precipitated material was washed 3 times in the

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cold with 5 per cent trichloroacetic acid and once with acetone at −10° C. The residue was then extracted 3 times with chloroform: methanol (2:1) at 45° to 50° C. The chloroform-methanol extract was washed 5 times with 2 vol of water, and sufficient methanol was added to the residual chloroform phase to obtain a clear solution. This solution was decolorized with activated charcoal and chromatographed on Florisil and on a mixed-bed ion exchange column as described by Radin and coworkers (10).

The cerebrosides obtained in this manner were recrystallized 3 times from methanol. Radioactivity was measured in a proportional gas-flow counter. The concentration of cerebrosides was determined by the anthrone reaction for hexoses after hydrolyzing the samples for 3 hours (10). The relative amounts of glucose and galactose in the hexose fractions were determined by incubating the cerebroside hexoses with glucose oxidase. After chromatography on an MB-3 (Rohm and Haas Co.) ion exchange column the galactose was determined and the amount of glucose was then calculated by the difference. Known amounts of glucose and galactose were carried as internal standards throughout the whole isolation procedure and the subsequent determination of galactose to ascertain the accuracy of these methods.

The total cerebroside content was estimated by the isotope dilution technique. A known amount of highly purified radioactive cerebroside obtained by tritiating, according to the method described by Wilzbach (11), was added to tissue homogenates, and the cerebrosides were isolated by the procedures outlined above. Comparison of the specific activity of the recovered cerebroside with that of the added cerebroside, as well as per cent recovery of the added isotope, permitted an accurate measurement of tissue cerebroside levels. The amounts of free glucose and galactose in tissue samples were estimated in trichloroacetic acid extracts of tissues by similar procedures with the use of labeled hexoses.

RESULTS

Accurate measurement of the specific activity of the cerebrosides obtained from the incubation mixtures required that the cerebrosides be obtained in a reasonably pure state. This restriction entailed considerable losses in the purification procedures which had to be determined. For this reason the isotope dilution technique was used to estimate total cerebroside content of samples of splenic tissue. Comparison of the data obtained by the isotope dilution method and colorimetric methods indicated that the latter measured from 31 to 58 per cent of the actual cerebroside content. Data of the levels obtained with the isotope dilution technique and the amount of glucose in the cerebroside hexoses are shown in Table I. The results indicate that considerable amounts of galactose were present in the cerebrosides of Gaucher's spleens as well as in the control spleens. Because the entire procedure was controlled with added standard amounts of glucose and galactose, these data are thought to be relatively reliable.

When the data of the incorporation of the isotopically-labeled precursors were compared, it became apparent that the uptake of each isotope by the tissue samples was essentially the same for all of the cases investigated in this study. The net incorporation of isotopes from the different precursors is shown in Table II. The data presented in the last column are expressed as specific activities of the recovered cerebrosides. These figures do not indicate that splenic tissue from patients with Gaucher's disease synthesized cerebrosides at a slower rate than did the control spleens.

TABLE I

Cerebroside contents of pathological states

Disease	Patient*	Age		Sex	Spleen weight	Total lipids†	Cerebroside content of spleen	Glucose in cerebroside hexoses
		yrs	*mos*		*g*	*% wet wt.*	*% wet wt.*	%
Gaucher's	K.S.[1]	6	2	♂	720	6.05	2.45	53
Gaucher's	S.R.[1]	4	1	♀	304	4.8	1.20	41
Gaucher's	C.R.[1]	5	8	♀	302	4.8	2.40	44
Gaucher's	J.A.[1]	8	10	♂	1,300	6.40	3.54†	32
Niemann-Pick	J.K.[1]	3	4	♂	258	12.9	1.05†	39
Niemann-Pick	C.C.[2]	5	6	♀	235	7.3	0.41	50
Idiopathic thrombocytopenic purpura	M.R.[3]	69		♀	73	‡	0.18	‡

* Superscripts indicate source of patient: [1] Children's Hospital Medical Center, Boston, Mass.; [2] Walter Reed Army Hospital, Washington, D. C.; [3] The George Washington University Hospital, Washington, D. C.

† These data courtesy Dr. Allen C. Crocker, Children's Hospital Medical Center, Boston, Mass.

‡ No analysis performed.

TABLE II

Incorporation of isotopic precursors into cerebrosides

Disease	Patient	Precursor	Incorporation into cerebrosides	
			cpm/g dry wt.	*mμmoles/ μmole*
Gaucher's	K.S.	Glucose-C^{14}	19,840	0.134
		Galactose-C^{14}	17,720	0.680
Gaucher's	S.R.	Glucose-C^{14}	4,210	0.051
		Galactose-C^{14}	6,960	0.163
Gaucher's	C.R.	Glucose-C^{14}	4,890	0.045
		Galactose-C^{14}	7,180	0.167
Gaucher's	J.A.	Galactose-C^{14}	19,800	0.054
		Acetate-C^{14}	21,660	0.120
Niemann-Pick	C.C.	Glucose-C^{14}	12,870	0.640
		Galactose-C^{14}	12,420	1.25
Niemann-Pick	J.K.	Galactose-C^{14}	12,700	0.95
		Acetate-C^{14}	15,500	3.05
Thrombocyt. purp.	M.R.	Glucose-C^{14}	1,850	0.326
		Galactose-C^{14}	4,960	1.087

Marked dilution of recovered isotope had taken place in the Gaucher's spleen by virtue of the larger amount of endogenous cerebrosides (cf. Table I).

Labeled galactose appeared on a molar basis to be a better precursor for cerebroside synthesis than is glucose. It was of considerable interest, therefore, to learn that appreciable portions of the galactose were subsequently isolated as glucose from the cerebroside hexoses (Table III). The amount of isotope derived from labeled hexoses in the ceramide (N-acyl sphingosine) portion of the cerebroside molecule did not exceed 13 per cent in the Gaucher's spleens. Such data might suggest that transglycosylation (12) had occurred during the incubation in contrast to *de novo* synthesis of the entire cerebroside molecule. Accordingly, the data obtained with labeled hexoses as precursors were compared with those found with labeled acetate. If the ceramide portion of the cerebroside molecule were synthesized during the incubation, rather than merely participating in an exchange of labeled hexose, one would expect a significant incorporation of labeled acetate into the sphingosine and fatty acid moieties. This indeed was found to be the case as was indicated by the significant incorporation of labeled acetate which was largely confined to the ceramide portion of the cerebroside molecule. The radioactivity in the ceramide was divided fairly evenly between the fatty acid moiety and sphingosine.

Because of some preference for galactose as a precursor for cerebroside hexoses, an investigation of the relative pool sizes of both free glucose and free galactose was undertaken in some of the tissues studied. The data shown in Table IV indicate that the apparent preferential incorporation of radioactivity from galactose into cerebrosides was not significantly influenced by the dilution of labeled precursor with endogenous glucose.

DISCUSSION

It is apparent that cerebroside synthesis in the spleens of patients with Gaucher's disease proceeds at a fairly active rate. The rate of cerebroside formation was essentially the same as that observed in a control spleen obtained from a patient with thrombocytopenic purpura and was almost identical with the values obtained in the spleens from two patients with Niemann-Pick disease.

TABLE III

Partition of isotopic label in cerebroside constituents

Disease	Patient	Substrate	Percent of label in:			
			Ceramides	Glucose	Hexoses	Galactose
Gaucher's	K.S.	Glucose-C^{14}	1.3	73		25
		Galactose-C^{14}	9	52		39
Gaucher's	C.R.	Glucose-C^{14}	13	75		12
		Galactose-C^{14}	13	59		28
Gaucher's	S.R.	Glucose-C^{14}	12		88	
		Galactose-C^{14}	13		87	
Gaucher's	J.A.	Galactose-C^{14}	6	60		34
		Acetate-C^{14}	59		41	
Niemann-Pick	C.C.	Glucose-C^{14}	10	22		68
		Galactose-C^{14}	13			
Niemann-Pick	J.K.	Galactose-C^{14}	49	35		16
		Acetate-C^{14}	95		5	

TABLE IV

Free hexose pools of pathological spleens

Disease	Patient	Total free hexose/g wet spleen	Glucose
		μmoles	%
Gaucher's	K.S.	2.31	81
Gaucher's	S.R.	3.27	68
Gaucher's	C.R.	4.05	61
Niemann-Pick	C.C.	3.90	36

Even though the possibility exists that some transglycosylation with radioactive hexose may have occurred during the incubation, the finding that radioactive acetate was well utilized as a precursor indicates that there was predominantly *de novo* synthesis of the entire cerebroside molecule.

Two findings were not anticipated at the outset of these investigations. First, the ratio of galactose to glucose in the cerebrosides of Gaucher's spleens was higher than expected from some of the values in the literature. Other investigators have reported that the cerebrosides from Gaucher's tissue contained mostly (4) or exclusively (5) glucose. Our observations do not agree with these findings, but seem to support the data obtained by Montreuil, Boulanger and Houcke (13), who reported a galactose: glucose ratio in cerebrosides of a case of Gaucher's disease as 0.80 in contrast to the ratio of 3.16 obtained in a normal spleen. Similarly, it is apparent that Lieb and Mladenović in an earlier study (14) had isolated a certain amount of galactose-cerebroside from a case of Gaucher's disease.

Second, the finding that galactose appears to be incorporated into cerebrosides at a higher rate than is glucose probably should not have been entirely unexpected, since normal tissue seems to synthesize predominantly the galactoside. Perhaps it is reasonable to assume that the enzymic mechanisms available for the utilization of galactose are preferentially engaged in this process in contrast with the multiplicity of pathways available for the metabolism of glucose. The relatively large amount of incorporated radioactivity from labeled galactose which had been converted to glucose, prior to or during the synthesis of the cerebroside molecule, is indicative of little, if any, change in the enzymes which catalyze the epimerization of uridinediphosphate galactose to uridinediphosphate glucose (15). Consistent with these observations is the fact that galactose tolerance is not impaired in Gaucher's disease (16).

From the data obtained in this study, it is concluded that the biochemical lesion leading to the lipodystrophic syndrome of Gaucher's disease probably does not lie in pathways concerned with the utilization of hexoses. Furthermore, it was shown that *de novo* synthesis of cerebrosides occurs at similar rates in Gaucher's cells and in the control tissues studied, although this does not necessarily imply that cerebosides in the spleen were solely derived by *de novo* synthesis. These observations suggest, however, that the nature of the biochemical defect may be catabolic rather than anabolic, and further investigations should be directed along lines of utilization and destruction of sphingolipids in lipodystrophic states.

SUMMARY

Cerebroside formation in slices of splenic tissue obtained from four patients with Gaucher's disease was compared with the rate observed in two cases of Niemann-Pick disease and one case of thrombocytopenic purpura. The biosynthesis of cerebrosides was examined with the use of labeled glucose, galactose, and acetate as substrates. Galactose was preferentially incorporated into the hexose moiety of the cerebrosides, although 40 to 60 per cent of the incorporated galactose was present as glucose-cerebroside. Significant quantities of galactose-cerebrosides were found in the Gaucher's spleens as well as glucose-cerebrosides. Cerebroside synthesis in Gaucher's disease does not appear to be sufficiently elevated to warrant the conclusion that this lipodystrophic condition is due to an increased rate of sphingolipid formation *in situ*.

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THE DIURETIC EFFECTS OF LARGE DOSES OF ACETAZOLAMIDE AND AN ANALOG LACKING CARBONIC ANHYDRASE INHIBITING ACTIVITY *

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According to current concepts, acidification of the urine in the distal tubule and final removal of bicarbonate from the glomerular filtrate occur by a process of ion exchange in which hydrogen ions within tubular cells are exchanged for sodium in the filtrate (1). It is thought that carbonic anhydrase catalyzes an essential reaction which either directly supplies hydrogen ions to the exchange site or removes the base generated by the secretion of hydrogen ions. In support of this hypothesis is the fact that unsubstituted sulfonamides which inhibit carbonic anhydrase prevent acidification of the urine and produce a diuresis of sodium and bicarbonate. Using sulfanilamide, Pitts and Alexander found that approximately 20 per cent of the filtered bicarbonate could be diverted into the urine (2), and suggested that the reabsorption of bicarbonate in the distal tubule was dependent on carbonic anhydrase activity.

Subsequently, however, it was pointed out by Berliner (3) and by Schwartz, Danzig and Relman (4) that acetazolamide, a more potent sulfonamide inhibitor of carbonic anhydrase, is capable of producing much greater effects on bicarbonate reabsorption. A dose of 20 to 30 mg per kg of acetazolamide resulted in the loss of up to 50 per cent of the filtered bicarbonate, thus suggesting that a sodium-hydrogen exchange catalyzed by carbonic anhydrase might be responsible for the reabsorption of bicarbonate in the proximal as well as distal tubules (3, 4). With intravenous doses of 500 mg per kg, even greater effects have been observed, with more than 75 per cent of the filtered bicarbonate appearing in the urine for a brief period after injection (5). Simultaneously, there was an enormous but transient diuresis, in which more than half of the filtered sodium and water was excreted.

These experiments were at first interpreted (4) as showing that the mechanism by which most, if not all, sodium reabsorption is accomplished involves the carbonic anhydrase-dependent hydrogen exchange described above. However, a more recent examination of this problem, using an N^2-methyl analog (CL 8490) of acetazolamide which has little or no enzyme-inhibiting activity, indicates that this drug also is a potent diuretic when given in comparably large doses (5). The purpose of the present report is to present in detail the experiments with high doses of acetazolamide as well as to report comparative data with the analog. The results are compatible with the view that a significant part of the diuretic action of large doses of acetazolamide resides in an additional property of the drug not related to carbonic anhydrase inhibition. It is probable, however, that further inhibition of enzyme also plays a role.

METHODS AND MATERIALS

Anesthetized dogs were used in all experiments. For the most part sodium pentobarbital was employed, but in some experiments morphine was administered. In some experiments the animals were curarized and artificially ventilated with 100 per cent oxygen using an intermittent

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positive pressure device (Mine Safety Pneophore) and in other experiments the animals were allowed to breathe room air spontaneously. In most experiments arterial P_{CO_2} ranged between 30 and 50 mm Hg, but in a few it was between 50 and 60 mm Hg. Continuous intravenous infusions of normal saline or saline and sodium bicarbonate were administered at rates necessary to maintain high urine flows and steady blood bicarbonate levels. Blood samples were collected from a catheter in the femoral artery, and infusions and all injections of drugs were made through a catheter in the femoral vein. The clearance of inulin or exogenous creatinine was used as a measure of glomerular filtration rate. Urine was collected under neutral mineral oil through an inlying bladder catheter. Inulin, pH, creatinine, sodium, potassium, CO_2 content and chloride were measured in blood and urine by standard methods, and P_{CO_2} and bicarbonate were calculated from the Henderson-Hasselbalch equation. A Donnan factor of 1.05 was used in the calculation of the filtered load of bicarbonate.

The usual experimental plan was as follows. After stable blood levels and urine flow were established, two or more 10-minute control periods were obtained. The desired dose of sulfonamide was then injected intravenously over a period of 2 to 3 minutes. The dosage levels of acetazolamide were 10 and 500 mg per kg, and equimolar quantities of the analog (CL 8490) were employed (10.6 and 536 mg per kg). Drugs were administered in volumes of 10 to 90 ml. The free compounds were put into solution by the addition of 1.8 mmoles sodium hydroxide per mmole of drug to make a final pH of approximately 8.5 to 9.0. In control experiments, sodium bicarbonate or sodium hydroxide was administered in amounts roughly equivalent to the alkali released into the blood by the larger doses of sulfonamide. Three minutes after the completion of each injection a blood sample was drawn, the bladder was emptied, the urine discarded and urine collections were begun anew. Because of the evanescent nature of the diuresis following the large doses, postinjection clearance periods were usually only 6 minutes in length.

The two sulfonamide compounds employed were: acetazolamide (Diamox, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide), and CL 8490 (N^2-methyl-5-acetamido-1,3,4-thiadiazole-2-sulfonamide.)[1] The latter substance has a titration curve which closely resembles that of acetazolamide. (Acetazolamide pKa'_1 7.4; pKa'_2 9.1; CL 8490 pKa'_1 7.7; pKa'_2 10.0). However, CL 8490 does not demonstrate significant carbonic anhydrase inhibitory activity either *in vitro* or *in vivo* (5). In several experiments blood and urine samples obtained just before and after injection of the sulfonamide were assayed by Dr. Thomas Maren for their carbonic anhydrase inhibitory activity by the enzymatic method (6, 7).

RESULTS

I. Comparative effects of equimolar small doses of CL 8490 (10.6 mg per kg) and acetazolamide (10 mg per kg). In five experiments 10.6 mg per kg of CL 8490 was directly compared with 10 mg per kg of acetazolamide. In all but one instance CL 8490 was injected first, followed in 1.5 to 2 hours by acetazolamide. In addition, CL 8490 was given alone on one occasion, and acetazolamide alone on two occasions. Table I summarizes the data from a typical experiment in which both drugs were given. Injection of CL 8490 produced little or no change in the renal excretion of water and electrolytes, and blood and urine contained no measurable carbonic anhydrase inhibiting activity. Subsequent injection of acetazolamide, on the other hand, was followed by a sharp rise in bicarbonate excretion and a reduction of approximately 40 per cent in the reabsorption of this ion. There was no

[1] This analog, synthesized by the American Cyanamid Company, was made available through the kindness of Dr. Thomas H. Maren of the Research Division.

TABLE I

Comparative effects of equimolar small doses of CL 8490 (10.6 mg/kg) and acetazolamide (10 mg/kg) *

Total elapsed time	Plasma					Urine						HCO_3 reabsorbed
	pH	P_{CO_2}	HCO_3	CAI activity†	GFR	Flow	Na	K	Cl	HCO_3	CAI activity†	
min		*mm Hg*	*mEq/L*	*μg/ml*	*ml/min*	*ml/min*		*μEq/min*			*μg/min*	*mEq/100 ml GFR*
60–70	7.32	38	20.0		70	5.1	955	52	975	26		1.96
70–80	7.32	39	20.2		70	5.1	992	49	995	27		1.98
80–90	7.31	41	20.3		72	4.7	949	47	951	27		1.99
90–92 CL 8490, 10.6 mg/kg i.v.												
95–105	7.31	42	21.6	0	70	4.5	932	41	926	37	0	2.11
105–115	7.32	40	21.3	0	75	4.6	984	44	960	56	0	2.06
115–190 Discard												
190–200	7.31	40	20.8	0	70	5.1	991	56	982	52	0	2.01
200–210	7.33	39	21.1		76	4.9	969	55	957	51		2.04
210–212 Acetazolamide, 10 mg/kg i.v.												
215–225	7.35	37	20.2	25	56	7.7	1,350	142	875	504	1,540	1.12
225–235	7.35	34	19.2	12	62	6.7	1,200	115	819	418	791	1.25

* Dog 131: weight 13.2 kg; sustaining infusion, 0.14 M NaCl at 8.5 ml/min beginning at 0 time. Control plasma, Na 140, K 2.6, Cl 112 mEq/L with no significant change throughout.

† Carbonic anhydrase inhibiting activity, expressed in terms of micrograms of acetazolamide.

TABLE II

Effect of 500 mg/kg of acetazolamide

Total elapsed time	Plasma							Urine					Excreted/filtered					HCO_3 reabsorbed
	pH	P_{CO_2}	HCO_3	Na	K	Cl	GFR	Flow	Na	K	Cl	HCO_3	H_2O	Na	K	Cl	HCO_3	
min		*mm Hg*		*mEq/L*			*ml/min*	*ml/min*	*μEq/min*						%			*mEq/100 ml GFR*
A. Dog Hazel: weight 22 kg. Sustaining infusion, NaCl 0.125 M, $NaHCO_3$ 0.025 M, at 9.6 ml/min at 0 time																		
120–130	7.43	37	23.6				81	8.0	1,140	67	800	292	9.9				15.2	2.00
130–140							80	8.0	1,140	63	800	280	10.1				14.9	2.01
140–150	7.41	38	23.4	157	2.6	118	83	8.4	1,190	71	848	286	10.1	9.6	32.9	8.7	15.0	1.99
150–158 Acetazolamide, 500 mg/kg i.v.																		
161–167	7.60	30	28.1	159	2.4	119	66	26.2	3,790	178	1,886	1,520	40.0	38.2	113.4	24.2	82.6	0.49
167–173	7.39	43	25.5	159	2.4	119	71	22.3	3,250	175	1,606	1,330	31.6	30.4	103.0	19.1	70.0	0.81
B. Dog 138: weight 12.7 kg. Sustaining infusion, NaCl 0.11 M, $NaHCO_3$ 0.035 M at 9.6 ml/min at 0 time																		
120–130	7.30	52	26.1	156	1.6	119	45	6.8	952	53	514	371	15.1	13.6	73.6	9.6	31.6	1.79
130–140	7.29	54	26.1			121	38	6.5	953	54	495	363	17.1	16.1	67.6	10.8	36.6	1.66
140–150	7.30	51	26.1	156	2.5	124	43	6.7	966	60	507	388	15.6	14.4	55.8	9.5	34.6	1.71
150–152 Acetazolamide, 500 mg/kg i.v.																		
153	7.68	29	34.9															
155–161	7.49	43	33.3	164	2.5	117	38	23.8	3,550	121	1,990	1,180	62.6	57.0	127.4	44.8	93.3	0.22
161–167	7.45	43	30.2			119	28	12.2	1,930	89	922	719	43.6	42.0	127.1	27.7	85.1	0.45
167–173	7.38	46	29.1	161	2.6	124	33	8.5	1,420	86	588	607	25.8	26.7	100.2	14.4	63.2	1.07

increase in chloride excretion and, although potassium excretion increased, most of the increment in bicarbonate was associated with a nearly equivalent rise in sodium excretion. Significant levels of carbonic anhydrase inhibiting activity were demonstrable in both plasma and urine. Urine flow increased slightly despite a drop in glomerular filtration rate.

Injection of 10.6 mg per kg of CL 8490 on five other occasions was similarly without effect on water and electrolyte excretion. Acetazolamide in this dose never failed to produce a significant reduction in bicarbonate reabsorption, but in no case increased chloride excretion.

II. The effects of 500 mg per kg of acetazolamide. In 15 experiments acetazolamide was administered intravenously at a dose of 500 mg per kg. On five occasions the drug was injected 2 to 3 hours after a comparable dose of analog, and in the remaining 10 experiments acetazolamide alone was injected. In Table II are summarized the results of two such experiments. In the first experiment (A), the dog had not received any other agent prior to injection of acetazolamide; in the other (B), a large dose of CL 8490 had been given approximately 90 minutes before, but its effect had almost entirely disappeared prior to injection of acetazolamide.

In both instances, injection of 500 mg per kg of acetazolamide resulted in a significant rise in blood pH and plasma bicarbonate concentration. There was also a very large diuresis of water and electrolytes. At the height of the diuresis in Experiment A, approximately 40 per cent of the water, 38 per cent of the sodium and 83 per cent of the bicarbonate in the filtrate was diverted into the urine. In Experiment B, the effect was even greater, with excretion of about 60 per cent of the filtered water and sodium and 90 per cent of the bicarbonate. In both experiments the diuresis was quite evanescent, for it was clearly waning after the first 6-minute clearance period. In both experiments there was also a large chloride and potassium diuresis, net secretion of the latter becoming demonstrable. The reabsorption of bicarbonate was briefly reduced to 0.49 mEq per 100 ml glomerular filtrate in A, and to 0.22 mEq per 100 ml glomerular filtrate in B. There was a slight reduction in glomerular filtration rate in both experiments.

In all the other experiments in which similar doses of acetazolamide were used the response was similar. The maximal rate of excretion of bicarbonate ranged from 70 to 95 per cent of the filtered load and the reduction in the rate of reabsorption ranged from approximately 45 per cent to 85 per cent of the control value, with an average of 65 per cent.

The maximal excretion of sodium and water was 20 to 57 per cent, and of chloride 13 to 45 per cent of the filtered load. As in the experiments in Table II, the glomerular filtration rate usually dropped slightly after the injection, but in a few cases there was no change. Qualitative tests of the urine for sugar in three experiments were negative. Phosphorus excretion, measured in sev-

TABLE III

Effects of 2 mmoles/kg of NaOH and $NaHCO_3$

Total elapsed time	Plasma							Urine					Excreted/filtered					HCO_3 reabsorbed
	pH	P_{CO_2}	HCO_3	Na	K	Cl	GFR	Flow	Na	K	Cl	HCO_3	H_2O	Na	K	Cl	HCO_3	
min		*mm Hg*		*mEq/L*			*ml/min*	*ml/min*		*μEq/min*					%			*mEq/100 ml GFR*
A. Dog 177, 12.9 kg. Sustaining infusion, 0.114 M NaCl plus 0.036 M $NaHCO_3$ and creatinine at 14 ml/min at 0 time.																		
144–154	7.35	51	32.5	153	2.7	105	52	4.8	1,120	42	636	349	9.2	14.2	30.0	11.6	20.7	2.58
154–164	7.41	49	31.2	153	2.9	103	48	3.8	903	41	546	310	7.9	12.3	29.5	11.1	20.7	2.48
165–167 NaOH, 26 mmoles in 90 ml H_2O i.v.																		
168–	7.54	42																
170–176	7.42	52	35.2	153	2.7	100	52	7.0	1,470	68	825	571	13.4	18.5	48.6	15.9	31.2	2.42
176–182	7.41	53	34.8	152	2.8	100	37	3.6	784	38	417	323	9.7	14.0	36.5	11.3	25.1	2.61
182–188	7.37	56	33.3	153	2.9	102	45	2.8	668	48	277	327	6.2	9.7	36.6	6.0	21.8	2.60
B. Dog 50, 11.4 kg. Sustaining infusion, 0.114 M NaCl + 0.036 M $NaHCO_3$ and creatinine at 14 ml/min at 0 time.																		
0–10	7.44	35	24.0	148	2.4	114	53	7.1	1,100	50	895	226	13.4	14.0	39.3	14.8	17.8	1.97
10–20	7.44	33	23.0	145	2.4	116	57	6.9	1,110	50	918	233	12.1	13.4	36.5	13.9	17.8	1.89
21–25 $NaHCO_3$, 23 mmoles in 75 ml H_2O i.v.																		
26–32	7.51	41	33.2	151	2.1	114	51	13.3	2,110	70	1,440	722	26.1	27.4	65.4	24.8	42.6	1.90
32–38	7.50	37	29.1	147	2.1	114	60	13.5	2,030	60	1,470	629	22.5	23.0	47.6	21.5	36.0	1.86
83–93	7.46	36	25.9	146	2.1	115	55	7.9	1,220	50	885	347	14.4	15.2	43.3	14.0	24.4	1.96
93–103	7.48	34	25.4	145	2.1	115	57	7.7	1,170	50	862	318	13.5	14.2	41.8	13.2	22.0	1.98

eral experiments, did not change. Urine and plasma osmolality were measured (by depression of freezing point) in five experiments. Values were in the range of 320 to 380 mOsm and were essentially equal in plasma and urine. Roughly estimated, either indirectly from the difference between total osmolality of the urine and the sum of the measured electrolytes or directly from the measurements of carbonic anhydrase inhibiting activity in the urine at the height of the diuresis (cf. Tables V and VI), the excretion of acetazolamide appeared to be less than 10 per cent of the total solute.

III. Sodium bicarbonate and sodium hydroxide. In order to determine whether any of the effects of acetazolamide could be attributed to the alkalinity of the infusate, experiments were carried out under comparable conditions, in which 2 mmoles per kg of NaOH or $NaHCO_3$ was given—an amount estimated to be at least equal to the alkali given up to the body fluids by the quantities of alkaline acetazolamide (or CL 8490) injected in the previous experiments.[2] Three experiments were carried out with sodium bicarbonate and four with sodium hydroxide. A summary of one of each type of experiment is shown in Table III. It can be seen that, although the injection of sodium hydroxide or sodium bicarbonate produced a rise in plasma bicarbonate concentration comparable with that effected by acetazolamide, there was a much smaller rise in the urinary excretion of sodium, chloride and water and very little increase in potassium excretion. Bicarbonate reabsorption was essentially unaffected although, as would be expected, the absolute excretion and the per cent of the filtered load excreted rose moderately.

IV. Effects of large doses of analog (CL 8490). In six experiments, 536 mg per kg of CL 8490 (equimolar with 500 mg per kg of acetazolamide) was administered intravenously. Table IV presents the results of an experiment in which 536 mg per kg of CL 8490 was injected approximately 90 minutes after injection of 10.6 mg per kg. It is evident that the low dose produced only a very slight effect on electrolyte excretion and no effect on the reabsorption of bicarbonate, as already shown in the experiment of Table I. With the large dose of analog, approximately 40 per cent of the filtered water, sodium and chloride appeared in the urine in association with two-thirds of the filtered bicarbonate. Bicarbonate reabsorption was transiently reduced by 50 per cent to a minimum rate of 0.9 mEq per 100 ml glomerular filtrate.

Tables V and VI summarize two experiments in which a large dose of CL 8490 was compared

[2] This quantity was calculated in the following manner. Acetazolamide has a pKa'_1 of 7.4 and a pKa'_2 of 9.1; the drug was injected at a pH of between 8.5 and 9.0 and was titrated by the blood to a pH of approximately 7.4 to 7.6. Therefore, it can be estimated that each buffer group would be titrated by approximately 0.5 equivalent per mole, or a total of 1 mEq per mmole of drug administered. Since the total dose of drug was approximately 2 mmoles per kg, the same load of NaOH or $NaHCO_3$ was administered. Similar considerations apply to CL 8490 which has virtually the same pK' values.

with an equimolar dose of acetazolamide. In the experiment of Table V, the injection of analog increased the rate of sodium excretion from 6 to 28 per cent of the filtered load and chloride excretion from 2 to 21 per cent of the filtered load. Urine flow rose from 6.1 to 19.7 ml per minute, and at the height of the diuresis 34 per cent of the volume of the filtrate was excreted. Bicarbonate excretion was 47 per cent of the filtered load, while the reabsorptive rate dropped 25 per cent. Subsequently, injection of acetazolamide resulted in a similar effect, with an increase in sodium excretion from 9 to 32 per cent of the load, in water from 8 to 33 per cent, and in chloride from 3 to 18 per cent. Bicarbonate excretion increased to a rate equal to 61 per cent of the filtered load and reabsorption was reduced by 37 per cent. Plasma and urine contained large quantities of carbonic anhydrase inhibiting activity following injection of acetazolamide, but never showed more than traces of activity after the analog.

The experiment in Table VI illustrates the more usual result, in that there was a distinctly greater effect on electrolyte excretion with acetazolamide than with CL 8490. With CL 8490 urine flow increased by 5 ml per minute, sodium excretion increased from 14 to 26 per cent of the filtered load, water from 13 to 28 per cent, chloride from 11 to 18 per cent, and bicarbonate from 30 to 55 per cent. Bicarbonate reabsorption was reduced by approximately 20 per cent. By contrast, after acetazolamide urine flow increased by 13 ml per minute and the maximal excretion of sodium rose from 13 to 40 per cent of the load, of bicarbonate from 34 to 75 per cent of the load, and of chloride from 8 to 27 per cent of the load. Bicarbonate reabsorption was reduced by approximately 55 per cent. As in the previous experiment, carbonic anhydrase inhibiting activity in urine was negligible immediately after administration of CL 8490, but very high after acetazolamide.

In Figure 1 is shown the maximal excretion of sodium following each of the experiments with acetazolamide and CL 8490. Sodium excretion, plotted as a per cent of the filtered load, is related to the simultaneous urine to plasma ratio for inulin or creatinine. Solid circles represent data obtained with acetazolamide and open circles, CL 8490. It is seen that maximal sodium excretion ranged from approximately 20 to 55 per cent of the filtered load with acetazolamide and from 20 to 40 per cent of the load with CL 8490. Although there was overlap, acetazolamide appeared to have a substantially greater effect. The heavy solid line in the figure represents the theoretical relationship between sodium and water excretion which would obtain if sodium were excreted isotonically. The close fit of the experimental points to this line indicates the virtually isotonic nature of the urine during diuresis. For comparison, the dotted lines in the lower part of the figure show the approximate range of the relationship between sodium and water observed during maximal osmotic diuresis with mannitol (8) and with urea (9). It is apparent that sodium excretion at any given rate of water excretion was considerably less with osmotic diuresis than with the drugs studied here. To the authors' knowledge, the maximal rates of sodium excretion observed in the

TABLE IV

Comparative effects of 10.6 mg/kg and 536 mg/kg of CL 8490 *

Total elapsed time	Plasma							Urine					Excreted/filtered					HCO_3 reabsorbed
	pH	P_{CO_2}	HCO_3	Na	K	Cl	GFR	Flow	Na	K	Cl	HCO_3	H_2O	Na	K	Cl	HCO_3	
min		*mm Hg*		*mEq/L*			*ml/min*	*ml/min*		*μEq/min*					%			*mEq/100 ml GFR*
50–60	7.42	29	19.0	149	3.3	121	65	5.9	1,070	76	1,110	60	9.1	11.0	35.4	14.1	4.9	1.81
60–70	7.38	32	19.4	149	3.4	123	63	5.0	1,110	81	1,140	63	9.4	11.8	37.8	14.7	5.2	1.84
70–80	7.38	32	19.6	148	3.5	122	64	6.1	1,120	89	1,180	66	9.5	11.8	39.7	15.1	5.3	1.86
80–81	CL 8490, 10.6 mg/kg i.v.																	
85–91	7.41	31	19.7	149	3.3	123	66	7.2	1,270	108	1,270	91	10.9	12.9	49.6	15.6	7.0	1.83
91–97	7.40	30	19.1	147	3.4	121	60	6.3	1,160	105	1,160	87	10.5	13.2	51.5	16.0	7.6	1.77
97–153	Discard																	
153–163	7.41	29	18.7	144	3.2	119	62	6.5	1,160	87	1,160	80	10.5	13.0	43.9	15.7	6.9	1.74
163–173	7.41	29	21.0	144	3.3	120	68	6.2	1,140	88	1,170	70	9.1	11.6	39.2	14.3	4.9	2.00
173–175	CL 8490, 536 mg/kg i.v.																	
175	8.00	11	28.5															
178–184	7.63	27	28.2	153	3.5	110	52	20.8	3,270	203	2,460	998	40.0	41.1	111.5	43.0	68.1	0.90
184–190	7.49	29	22.3	154	3.0	118	52	12.5	2,120	153	1,700	541	24.0	26.5	98.1	27.7	46.6	1.19

* Dog 127: weight 12.8 kg; sustaining infusion, 0.15 M NaCl at 8.5 ml/min at 0 time.

TABLE V

Comparative effects of equimolar large doses of CL 8490 (536 mg/kg) and acetazolamide (500 mg/kg) *

Total elapsed time	Plasma						CAI activity†	GFR	Urine					Excreted/filtered					HCO_3 reabsorbed	CAI activity†
	pH	P_{CO_2}	HCO_3	Na	K	Cl			Flow	Na	K	Cl	HCO_3	H_2O	Na	K	Cl	HCO_3		
min		*mm Hg*		*mEq/L*			*μg/ml*	*ml/min*	*ml/min*	*μEq/min*						*%*			*mEq/100 ml GFR*	*μg/min*
120–130	7.38	64	39.0	158	2.7	115		50	5.2	392	33	108	300	10.4	5.0	24.4	1.9	15.4	3.30	
130–140	7.40	61	38.6	158	2.7	115	0	56	6.1	512	39	149	354	10.9	5.8	25.8	2.3	16.4	3.23	0
140–142 CL 8490, 536 mg/kg i.v.																				
145–151	7.64	44	45.5	164	3.1	113	2	58	19.7	2,620	108	1,350	1,250	34.0	27.5	60.1	20.6	47.4	2.40	40
151–157	7.52	55	43.5	164	2.9	113	2	48	13.7	1,730	87	825	936	28.5	22.0	62.5	15.2	44.8	2.40	
157–298 Discard																				
298–308	7.49	47	36.1	158	2.8	119	4	44	3.6	583	54	111	473	8.2	8.4	43.8	2.1	27.5	2.62	61
308–318	7.43	53	38.1	158	2.8	119		49	4.1	689	67	146	520	8.4	8.9	48.8	2.5	27.9	2.75	
318–320 Acetazolamide, 500 mg/kg i.v.																				
323–329	7.65	39	43.3	160	3.0	118	1,700	54	18.0	2,750	150	1,160	1,430	33.3	31.8	92.6	18.2	61.2	1.68	40,500
329–335	7.49	52	40.2	162	2.6	116	950	42	13.3	2,060	80	959	988	31.7	30.3	81.5	19.7	58.5	1.67	20,615

* Dog 120: weight 16.4 kg; sustaining infusion, 0.06 M $NaHCO_3$ + 0.08 M NaCl at 8.5 ml/min starting at 0 time.
† Carbonic anhydrase inhibiting activity, expressed in terms of acetazolamide.

TABLE VI

Comparative effects of equimolar large doses of CL 8490 (536 mg/kg) and acetazolamide (500 mg/kg) *

Total elapsed time	Plasma						GFR	Urine						Excreted/filtered					HCO_3 reabsorbed	CAI activity†
	pH	P_{CO_2}	HCO_3	Na	K	Cl		Flow	Osmol.	Na	K	Cl	HCO_3	H_2O	Na	K	Cl	HCO_3		
min		*mm Hg*		*mEq/L*			*ml/min*	*ml/min*		*μEq/min*						*%*			*mEq/100 ml GFR*	*μg/min*
50–60	7.46	44	32.2	151	3.4	115	57	6.9	367	1,140	125	610	566	12.1	13.2	64.5	9.3	30.8	2.23	
60–70	7.46	42	30.7	151	3.0	116	45	6.1	379			573	528	13.6			11.0	38.2	1.90	
70–80	7.46	42	30.7	151	3.0	116	56	7.3	368	1,210	123	696	523	13.0	14.3	73.2	10.7	30.4	2.14	0
80–91 CL 8490, 536 mg/kg i.v.																				
91–97	7.61	36	38.0	172	2.6	111	44	12.2	327	1,930	172	864	911	27.7	25.5	150.3	17.7	54.5	1.73	22
97–103	7.55	37	33.3			112	45	9.2	347	1,540	118	800	662	20.4			15.9	44.2	1.86	23
103–109	7.53	37	32.4	159	2.2	112	45	10.5	337	1,710	118	966	691	23.3	23.9	119.2	19.2	47.4	1.70	38
109–192 Discard																				
192–202	7.42	42	27.7	158	2.9	122	47	7.3	297	986	106	451	495	15.5	13.3	77.8	7.9	38.0	1.72	104
202–212	7.42	42	27.7	158	2.9	122	47	7.0	294	952	96	475	440	14.9	12.8	70.4	8.3	33.8	1.83	106
212–214 Acetazolamide, 500 mg/kg i.v.																				
214–217 Discard																				
217–223	7.45	47	33.9	164	3.2	119	47	20.5	299	3,100	203	1,530	1,200	43.6	40.2	135.0	27.4	75.3	0.84	68,700
223–229	7.43	46	31.7			120	43	12.5	314	1,960	149	923	836	29.1			17.9	61.3	1.23	35,600
229–235	7.43	45	30.7	164	2.9	121	42	10.3	315	1,640	128	770	713	24.5	23.8	105.1	15.2	55.3	1.37	30,900

* Dog 140: weight 13 kg; sustaining infusion, 0.11 M NaCl and 0.03 M $NaHCO_3$ at 10 ml/min starting at 0 time.
† Carbonic anhydrase inhibiting activity, expressed in terms of acetazolamide.

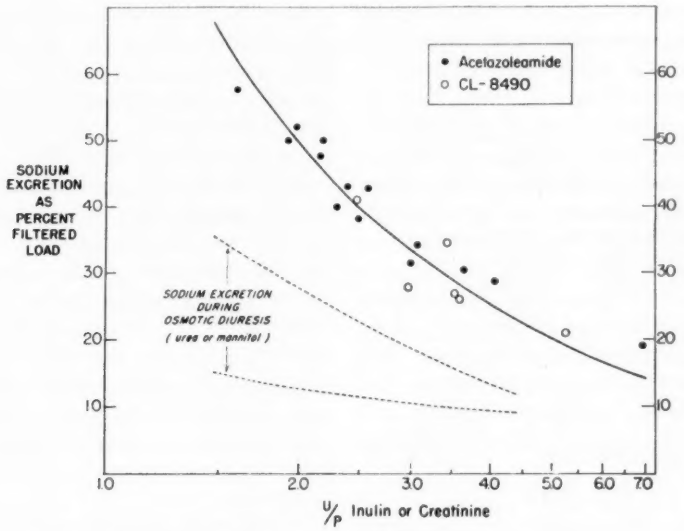

FIG. 1. MAXIMAL SODIUM EXCRETION FOLLOWING ACETAZOLAMIDE (500 MG PER KG) OR CL 8490 (536 MG PER KG). This is plotted as a per cent of the filtered load in relation to the simultaneous urine to plasma ratio for inulin or creatinine. Solid dots represent acetazolamide, open circles CL 8490. The heavy solid line represents the theoretical relationship between these parameters for the excretion of an isotonic solution of sodium. Note that the experimental points closely fit this theoretical line. For comparison, the dotted lines in the lower part of the figure indicate the range of the relationship between excretion of sodium and water during maximal osmotic diuresis with mannitol (8) and urea (9).

present study exceed any which have previously been reported.

DISCUSSION

These experiments show that rapid intravenous administration of 500 mg per kg of acetazolamide produces a large, though transient, diuresis which considerably exceeds that which has been produced by any other experimental technique. At the height of this effect as much as 40 to 60 per cent of the sodium and water in the glomerular filtrate was diverted into the urine, together with 25 to 50 per cent of the filtered chloride. Bicarbonate reabsorption was depressed by 40 to 85 per cent from control rates and the rate of excretion was as much as 70 to 95 per cent of the filtered load. Aside from its magnitude, the feature of this diuresis which distinguished it from the effects of the usual small doses of acetazolamide was the massive chloruresis. When 10 mg per kg was given, there was little or no effect on chloride, and almost all of the increment in anion was due to bicarbonate (Table I). By contrast, in the present experiments with 500 mg per kg, although there was a further enhancement of bicarbonate excretion, a large part of the increased excretion of anion was due to chloride.

The explanation for this phenomenon was at first thought to reside solely in the carbonic anhydrase inhibiting activity of the large dose (4). It would now appear that an additional mechanism must be invoked because equimolar large doses of an inactive analog (CL 8490) also produce a substantial diuresis of electrolyte and water. This diuresis is not usually as large as that resulting from acetazolamide, but closely resembles the latter in its duration and in its effect on urine composition.

It seems clear from the data at hand that neither the osmotic effect nor the acute systemic alkalinizing action of the large dose of the sodium salts of acetazolamide or CL 8490 can explain the results.

An osmotic diuresis would appear to be excluded by the fact that the drug constituted only a small fraction of total urine solute, probably less than 10 per cent. That neither the sodium load nor the extracellular alkalinizing effect of the sulfonamide base could account for the depression of chloride and bicarbonate reabsorption was demonstrated by control experiments in which loads of sodium bicarbonate and sodium hydroxide equivalent to the alkali yielded by the sulfonamides were found not to reduce the reabsorption of bicarbonate and to only slightly increase the excretion of sodium and chloride. Administration of the alkali produced a moderate bicarbonate diuresis, but this was due to the rise in plasma bicarbonate level and not to any significant reduction in reabsorption.

What then is the explanation for the diuretic action of CL 8490 and for that part of the action of the large dose of acetazolamide which presumably is not related to carbonic anhydrase inhibition? The available data do not permit a definite answer to this question. However, in view of the large doses required to produce diuresis and the transient nature of the effect, it is tentatively suggested that the mechanism does not involve direct inhibition of an enzymatic process required for electrolyte transport. Rather, it would appear more likely that the effect depends upon some temporary change in renal cell composition resulting from the sudden accumulation of large amounts of drug. There is evidence that acetazolamide is both secreted and reabsorbed by tubular cells (10) and it is reasonable to assume similar handling of the analog. Movement of the monobasic form of either drug from the relatively alkaline plasma or proximal tubular fluid (pH 7.5 to 7.7) into the relatively acid renal cells (estimated pH < 7.0) would result in binding of intracellular protons by the weaker of the two basic groups on each molecule (pK'_a 7.4 and 7.7, respectively) and as a consequence cell pH would rise. Since there is strong evidence to suggest that reabsorption of bicarbonate involves a sodium-hydrogen exchange (1), acute reduction in the availability of protons within the tubular cell might inhibit this process and produce a bicarbonate diuresis. Whether a similar sodium-hydrogen exchange is also involved in the reabsorption of chloride (3) is not known, but if such were the case, a rise in cellular pH would also be expected to reduce the reabsorption of chloride. If, on the other hand, a sodium-hydrogen exchange does not precede chloride reabsorption, it might be postulated that increased cell pH in some manner slows the metabolic processes upon which all active sodium transport depends. Since chloride reabsorption appears to be a passive result of active sodium transport (11), the final results would be the same. The transient nature of the diuretic effect, assuming the latter to be the result of abrupt changes in cell pH, could be explained on the basis of the brief time during which plasma and tissue concentrations of drug might remain at levels high enough to affect cell pH, and to rapid turnover of protons within the cell, which would be expected to restore the usual pH very quickly and thus nullify the diuretic effect.

While such speculation seems consistent with available data, it is obvious that alternative mechanisms cannot be excluded. Thus, for example, it is possible that the diuretic action shared by acetazolamide and CL 8490 resides in some other property of the thiadiazole-sulfonamide configuration which also belongs to the benzothiadiazole-sulfonamides of the chlorothiazide family. The latter are potent saluretics which in relatively low dosage produce changes in electrolyte excretion qualitatively similar to those reported here (12, 13). More information will be needed to settle this question.

Finally, it should be emphasized that, in three of five paired experiments, acetazolamide appeared to have a considerably greater effect on sodium and water than did CL 8490. The maximal diuresis achieved with CL 8490 never approached that observed in other experiments with acetazolamide (Figure 1). It is likely, therefore, that an appreciable part of the action of the 500 mg per kg dose of acetazolamide is in fact due to carbonic anhydrase inhibition. Limited supplies of CL 8490 unfortunately prevented a more definitive quantitative comparison of acetazolamide with CL 8490.

SUMMARY AND CONCLUSIONS

Intravenous injection of 500 mg per kg of sodium acetazolamide in dogs produced a massive but transient diuresis in which as much as 40 to 60 per cent of the filtered sodium and water and 25

to 50 per cent of the filtered chloride were diverted into the urine. Bicarbonate reabsorption was depressed by 40 to 85 per cent from control rates and the rate of excretion reached 70 to 95 per cent of the filtered load. A substantial part of this effect is probably not explained by inhibition of carbonic anhydrase, because the sodium salt of the N^2-methyl analog of acetazolamide (CL 8490), which has virtually no carbonic anhydrase inhibiting activity *in vivo* or *in vitro,* was found to produce a similar though somewhat smaller diuresis.

The data indicate that the diuretic action shared by the sodium salts of acetazolamide and CL 8490 cannot be explained on the basis either of the osmotic activity of the administered drugs or their extracellular alkalinizing effects. In view of the size of the dose required and of the transiency of the effect, it is proposed that the shared mechanism of action depends upon some temporary alteration in renal cell composition. It is tentatively suggested that depression of electrolyte reabsorption may result from transient intracellular alkalosis produced by cellular accumulation of the monobasic form of the drug.

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STUDIES ON THE SYNTHESIS AND SECRETION OF SERUM LIPOPROTEINS BY RAT LIVER SLICES

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There is at present a great deal of interest in factors determining the levels of serum lipids because of their possible relation to atherosclerosis. Since the serum lipids circulate only in the form of lipid-protein complexes, the mechanism of production of these complexes and their delivery into and removal from the serum must be understood before there is a sound basis for defining the homeostatic mechanisms controlling the levels of individual lipid components. While factors influencing the metabolism of any one of the individual lipid moieties may influence the serum levels of that moiety, such an effect must ultimately be expressed indirectly through an alteration in the rate or pattern of metabolism of the lipid-protein complexes of which it is a part.

The present experiments were undertaken in order to define an *in vitro* system in which lipoprotein biosynthesis could be studied without the many complicating variables encountered in whole-animal studies. It is generally accepted that the liver is the principal source of the serum lipids (1–3) and it has been assumed, but not proved, that the liver is also the source of the protein moiety of the serum lipoproteins. We have previously described the incorporation by perfused rat liver and by rat liver slices of labeled amino acids into proteins with densities similar to those of the serum lipoproteins (4, 5). The studies reported here provide evidence that the high density lipoproteins synthesized and secreted by rat liver slices are identical with the high density lipoproteins of normal rat serum. The time course of synthesis of both the protein and the lipid moieties in the total lipoprotein fraction, the rate of appearance of radioactive lipoprotein in the medium, and the net changes in protein and lipid in liver slice and medium are presented. The effects of cholesterol feeding, Triton injection and the nephrotic state are also described.

MATERIALS AND METHODS

DL-leucine-1-C^{14} (5.3 mc per mmole) and sodium acetate-1-C^{14} (3.5 mc per mmole) were obtained from the Nuclear-Chicago Corp. A complete mixture of uniformly labeled C^{14}-amino acids was prepared from a thermophilic strain of *Chlorella* grown in a synthetic medium with $C^{14}O_2$ as the sole source of carbon, according to the method described by Catch (6). The cells were lysed and the lipids were extracted with organic solvents. The precipitated proteins were washed several times with 10 per cent trichloroacetic acid (TCA) and then with organic solvents. The washed proteins were hydrolyzed in 5.5 N HCl, the excess HCl was removed by evaporation to dryness, and the hydrolysate was passed over a column of Dowex-50 in the H^+ form. The amino acids, eluted with 4 M NH_4OH, had a specific radioactivity of 25 mc per mmole of ninhydrin color (referred to leucine).

Twice-recrystallized trypsin and chymotrypsin were obtained from Armour Laboratories. Triton WR-1339 (oxyethylated tertiary octyl phenol formaldehyde polymer) was obtained from Winthrop Laboratories.

Tissue incubations. Male Osborne-Mendel rats, maintained on Purina Laboratory Chow *ad libitum* and weighing approximately 250 g at the time of sacrifice, were used. Rats with the nephrotic syndrome were prepared [1] by the injection of nephrotoxic serum using the methods described by Baxter and Goodman (7). The rats were decapitated, the livers were rapidly removed and chilled, and slices were prepared for incubation using a Stadie-Riggs microtome. In most experiments 500 mg of liver slices was incubated in 5 ml of fresh rat serum prepared from blood drawn from control rats under ether anesthesia. The slices were incubated for up to 4 hours at 37° C under an atmosphere of 95 per cent O_2–5 per cent CO_2. In one set of experiments, in order to permit measurement of net changes in the medium, liver slices were incubated in Krebs' bicarbonate medium.

Isolation of lipoprotein fractions. At the end of the incubation the liver slices were removed from the medium which was then adjusted to the desired density. In order

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[1] We acknowledge with thanks the assistance of Dr. James C. Allen and Dr. James H. Baxter in making these animals available.

to minimize solution volumes, adjustments of density were made by addition of the appropriate amounts of solid KBr calculated according to the following equation:

$$V_o d_o + x = d(V_o + \bar{V}x),$$

where x = amount of KBr in grams, V_o = initial volume in milliliters, d_o = initial density, d = final (desired) density, and $\bar{V}$ = apparent specific volume of KBr at the final density (8).

In studies of the time course of lipoprotein synthesis aliquots of tissue slices and medium were removed at intervals. The tissue slices were homogenized in 0.9 per cent saline in a glass homogenizer. These saline homogenates and the aliquots of medium were centrifuged for 1 hour at 100,000 × G in a Spinco model L preparative ultracentrifuge. Lipids floating to the top of the tube were removed with a tube slicer (9) and the clear supernatant was decanted, leaving behind cellular debris. The supernatant solution was then adjusted to density 1.21 and centrifuged for 48 hours at 114,000 × G. The upper 2.5 ml of the tube volume was isolated by means of the tube slicer. In the course of these studies it was found that, although all of the lipoproteins were concentrated into the upper 2.5 ml of the centrifuge tube by 48 hours of centrifugation at 114,000 × G, the layer immediately below the lipoproteins was not completely free of protein. This may reflect the presence of proteins with density close to 1.21 or the presence of tissue debris with density near 1.21, possibly fragments of cell wall. The presence of such non-lipoprotein proteins extending into the upper layer would alter the observed specific radioactivities for the lipoprotein fractions. To minimize possible errors of this kind the serum medium in the later studies was adjusted to density 1.21 and carefully layered under KBr solution of equal density so that the lipoproteins migrated to the top of the tube through a protein-free medium. In addition, the isolated lipoprotein fractions were passed through Millipore filters (0.4 μ pore diameter) to remove any particles of low density cell debris prior to analysis.

In the studies designed to characterize the protein moieties and relate them to serum lipoproteins the serum medium was first adjusted to density 1.063 and centrifuged at 114,000 × G for 24 hours. The low density lipoproteins (*$D < 1.063$ fraction*) were separated and the infranatant solution was adjusted to density 1.21. After centrifugation for 48 hours at 114,000 × G the upper layer (*D 1.063–1.21 fraction*) was separated. The proteins in the infranatant layer, presumably free of lipoproteins, are referred to as the *$D > 1.21$ fraction* or the *residual proteins.*

Proteolytic degradation and characterization by peptide pattern. The work of Sanger and Tuppy (10) and that of Ingram (11) has shown that the digestion of proteins with pure proteolytic enzymes of well defined specificity results in the production of a highly reproducible mixture of peptide degradation products. These usually can be well separated on paper using chromatography in one direction and high-voltage electrophoresis in the perpendicular direction. The resulting peptide pattern, developed by the use of ninhydrin staining, is characteristic for each protein and has been termed a "fingerprint" by Ingram (11). We employed a modification of this method [2] described by Katz, Dreyer and Anfinsen (12).

The ultracentrifugally isolated lipoproteins were delipidated by extraction with acetone-ethanol and then ether. The delipidated protein was suspended in 0.2 ml of 6 M urea in 0.2 M NH_4HCO_3 at pH 8.2 and allowed to remain overnight at 4° C. Then 2 vol of 0.2 M NH_4HCO_3 was added to lower the urea concentration to 2 M, and 25 μg of trypsin was added. After incubation for 2 hours at 30° C, 12.5 μg of chymotrypsin was added, and the incubation was continued for an additional 4 hours. The digest was then passed over a column of Dowex-50 (2 per cent cross-linkage; 50 to 100 mesh) in the H^+ form, and several column volumes of water were passed through. The peptides were eluted with 4 M NH_4OH and the eluate was lyophilized. Aliquots of the peptide mixture were spotted on sheets of Whatman no. 3 filter paper and the peptides were separated along one axis by descending chromatography using *n*-butanol : acetic acid : water (4 : 1 : 5) and then by high-voltage electrophoresis (13) in the perpendicular axis. Because the electrophoresis tank could not accommodate the full sheet it was necessary to cut each chromatogram in half and run the electrophoresis in two stages. Fingerprints obtained from lipoproteins synthesized in the presence of a complete mixture of C^{14}-labeled amino acids were autoradiographed by placing them in contact with Eastman Kodak type KK industrial X-ray film in cassettes. These were stored at − 15° C for 1 to 2 months at the end of which time the film was developed by standard methods.

Measurement of protein and protein radioactivity. Protein in the fractions analyzed was precipitated with 5 per cent TCA, washed once with 5 per cent TCA at 90° C, twice with cold TCA, once with ethanol : acetone, 1 : 1 (vol/vol), at 60° C, twice with cold 1 : 1 ethanol : acetone and once with ether. In the case of the $D > 1.21$ fraction, the TCA precipitate was resuspended and adjusted to a pH of approximately 6 before addition of ethanol-acetone because of the appreciable solubility of serum albumin in acidic ethanol-acetone. The dried protein precipitate was dissolved in 1 N NaOH. One aliquot of the alkaline solution was used for measurement of total protein by the method of Lowry, Rosebrough, Farr and Randall (14). For measurement of the small amounts of lipoprotein-protein in incubation media (less than 20 μg) it was necessary to scale this method down, using the Beckman spectrophotometer with microcell attachment for readings of optical density. A second aliquot was transferred to a counting vial and dried at 130° C for 4 hours. One ml of 1 N Hyamine in methanol (Rohm and Haas, Philadelphia, Pa.) was added to the dried cake in the bottom of the vial and it was allowed to stand overnight at room temperature. The next day the remaining caked material was broken up with a spatula, and Hyamine remaining on the spatula was rinsed back

[2] We acknowledge with thanks the generous assistance of Dr. Arnold Katz in our application of this technique.

into the vial with methanol. The vial was then heated at 40 to 60° C for 4 hours, after which 15 ml of 0.6 per cent 2,5-diphenyloxazole in toluene was added with 1 to 2 ml additional methanol. This procedure leaves a residual white sediment in the vial, presumably of NaOH, but the extraction of labeled protein by Hyamine appears to be complete (15). This was demonstrated in the range from 10 to 200 μg of protein sample with a sample of C^{14}-labeled protein of known specific radioactivity. Radioactivity was measured in a Packard liquid scintillation spectrometer.

Measurement of lipids and lipid radioactivity. The pooled ethanol-acetone and ether extracts were taken to dryness on a steam bath and redissolved in chloroform: methanol, 2:1 (vol/vol). The chloroform-methanol extract was washed three times according to the method of Folch and co-workers to remove nonlipid materials (16). An aliquot for radioassay was taken to dryness in a counting vial to which was added 15 ml of 0.6 per cent 2,5-diphenyloxazole in toluene, and the vial was counted in the liquid scintillation spectrometer. In some experiments the incorporation of acetate-1-C^{14} into the nonsaponifiable lipid fraction was measured (17). Cholesterol was determined by the method of Sperry and Webb (18) and phospholipids were determined by the method of Stewart and Hendry (19).

RESULTS

Identification of newly synthesized and secreted D 1.063–1.21 lipoproteins with normal serum D 1.063–1.21 lipoprotein. The net amount of lipoprotein appearing in an inorganic medium during liver slice incubations was extremely small—too small to permit direct chemical and physical characterization. An indirect but highly specific method was used to show that the newly synthesized D 1.063–1.21 lipoproteins were identical with those normally present in rat serum. The approach used was based on the uniqueness of the peptide pattern obtained by digestion of a protein with pure proteolytic enzymes of well defined specificity (10–12). Newly synthesized lipoproteins labeled throughout the peptide chain were prepared by incubating rat liver slices in serum with a complete mixture of uniformly C^{14}-labeled amino acids of high specific radioactivity. If the newly synthesized D 1.063–1.21 lipoproteins are structurally identical with the normal serum lipoproteins in that density class, then there should be radioactivity associated with every peptide derived from the serum lipoproteins, and the radioactivity in the peptide mixture should be limited exclusively to these peptides. The peptide pattern or fingerprint, as developed with ninhydrin staining, demonstrates exclusively the peptides derived from the lipoproteins in the serum incubation medium; the quantities of lipoprotein added to the serum medium during incubation are negligible relative to the quantities in the serum. In other words, the lipoproteins in the serum medium serve here as unlabeled carrier material for the small amounts of highly labeled lipoprotein produced during the incubation.

In Figure 1b is shown a photograph of the ninhydrin-positive spots in a fingerprint of the D 1.063–1.21 lipoprotein isolated from the serum medium after incubation of liver slices with C^{14}-labeled amino acids. In Figure 1c is shown a fingerprint of D 1.063–1.21 lipoprotein of normal rat serum. Comparison of these indicates the reproducibility of the peptide pattern and also shows that the incubation with liver slices did not introduce any detectable amounts of protein differing structurally from those present in this class in normal serum. In Figure 1a is shown a photograph of an autoradiogram made from the fingerprint shown in Figure 1b. While the resolution in the central group of peptides in the autoradiogram is not adequately shown, direct comparison with the fingerprint shows that to every peptide spot developed with ninhydrin there is a corresponding radioactive spot developed on the autoradiogram. Furthermore, there are no radioactive spots not associated with a corresponding ninhydrin spot. It should be noted that the *intensities* of the spots in the autoradiogram are not expected to parallel the intensities of the ninhydrin spots. Assuming stoichiometric splitting of susceptible bonds, the intensities of the spots in the autoradiogram will be roughly proportional to the lengths of the peptides, since all the amino acids are labeled. On the other hand, the ninhydrin color yield of the peptides will depend upon the number of free amino groups and the particular amino acid composition. Only the locations of the spots should be considered. It can be concluded that the newly synthesized D 1.063–1.21 lipoprotein-proteins secreted into the medium are structurally identical with normal rat serum D 1.063–1.21 lipoprotein-proteins.

Peptide pattern of D < 1.063 lipoproteins. A peptide pattern and autoradiogram were prepared from labeled D < 1.063 lipoproteins prepared ex-

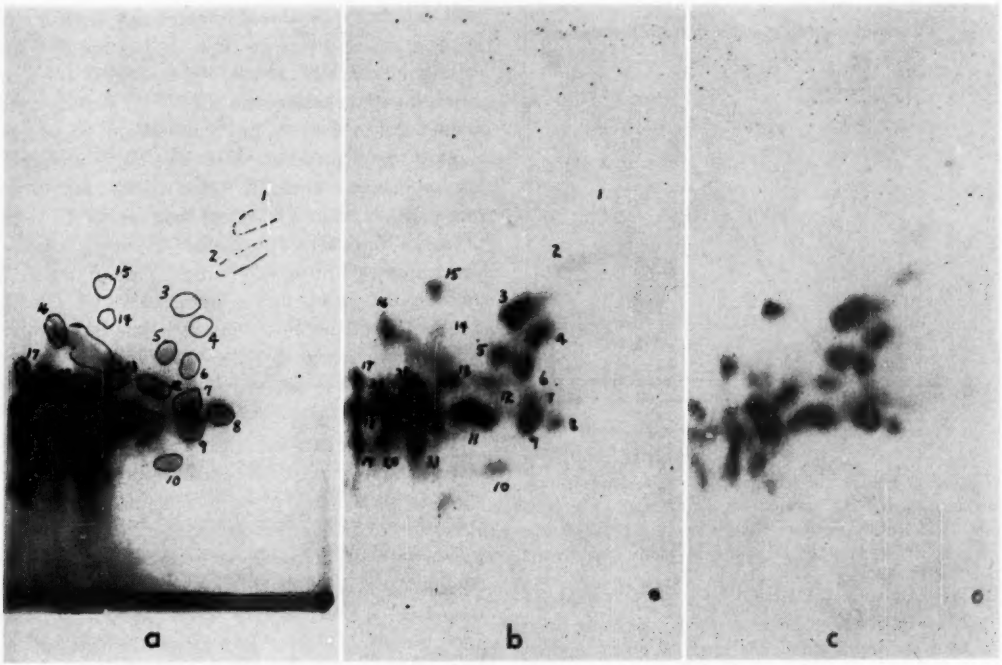

FIG. 1. a) AUTORADIOGRAM OF PEPTIDE PATTERN SHOWN IN b. The better-defined spots in the X-ray film have been outlined on an overlay and numbered to indicate correspondence with ninhydrin-positive spots in the fingerprint. b) PEPTIDE PATTERN OR "FINGERPRINT" OBTAINED FROM D 1.063–1.21 LIPOPROTEIN ISOLATED FROM THE SERUM MEDIUM AFTER INCUBATION FOR 4 HOURS AT 37° C WITH RAT LIVER SLICES AND A COMPLETE MIXTURE OF RANDOMLY LABELED C^{14}-AMINO ACIDS AS DESCRIBED IN THE TEXT. (Paper chromatography in the horizontal direction, high voltage electrophoresis in the vertical direction.) A few peptide spots, which ran near the front, are not shown since the paper had to be divided prior to electrophoresis. These spots not shown also correlated well with radioactive spots in the autoradiogram. c) PEPTIDE PATTERN OF D 1.063–1.21 LIPOPROTEIN-PROTEIN ISOLATED FROM NORMAL RAT SERUM THAT HAD BEEN INCUBATED AS ABOVE BUT IN THE ABSENCE OF LIVER SLICES.

actly as described above for the D 1.063–1.21 lipoproteins. There were approximately 24 peptide spots in the peptide pattern as developed with ninhydrin. The pattern of these peptides isolated from the serum medium at the end of incubation with liver slices was indistinguishable from that obtained from D < 1.063 lipoproteins isolated from normal rat serum. Thus, as in the case of the D 1.063–1.21 lipoproteins, there was no evidence for the addition of significant amounts of new D < 1.063 lipoproteins to the medium as the result of incubation with liver slices.

Autoradiography revealed the presence of radioactivity in 17 of the 24 detectable ninhydrin-staining peptides, but there were 10 to 15 radioactive spots not correlated with a ninhydrin positive spot. The incomplete identity between peptide pattern and autoradiogram may be due to technical difficulties such as incomplete proteolytic degradation or inadequate exposure time. Alternatively, the result may truly indicate that the newly synthesized lipoproteins appearing in the medium are not identical with or represent only a small fraction of the D < 1.063 lipoproteins found in normal rat serum. The presence of radioactivity in the large majority of the peptides derived from circulating low density lipoprotein suggests, but does not prove, that some of the newly synthesized and secreted lipoproteins do represent normal low density serum lipoproteins. Support for this interpretation comes from the studies of Marsh and Whereat, reported while this work was in progress (20, 21). These authors, using immunological techniques, have shown that rat liver slices incubated in an inorganic medium secrete low density lipoproteins that are im-

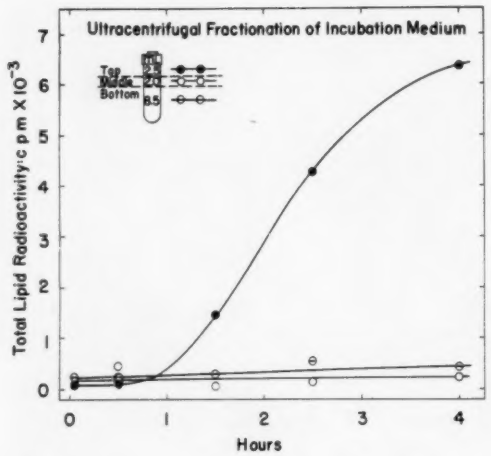

FIG. 2. TOTAL LIPID RADIOACTIVITY IN MEDIUM LIPOPROTEINS ($D < 1.21$) AS A FUNCTION OF TIME OF INCUBATION. Rat liver slices were incubated in Krebs' bicarbonate buffer containing 1-C^{14}-acetate and 1-C^{14}-leucine. Upper left: the key here indicates the manner in which the centrifuge tube was divided for analysis. After centrifugation as described under Methods, the upper 2.5 ml was taken as one fraction, the next 2.0 ml as a second fraction and finally the bottom 8.5 ml as a third fraction. Analyses of these fractions are shown separately according to this key in Figures 2, 3 and 5.

munochemically the same as low density lipoproteins found in normal rat serum.

Time course of lipoprotein synthesis. In all of the following studies the total lipoprotein fraction ($D < 1.21$) was isolated without subfractionation into low and high density groups. When rat liver slices were incubated in bicarbonate buffer with 1-C^{14}-acetate and 1-C^{14}-leucine, lipoproteins labeled both in the lipid and protein moieties appeared in the medium. In zero time control samples, there was negligible radioactivity in both lipid and protein moieties. As shown in Figure 2 there was an initial lag period in the appearance of labeled lipid in the lipoproteins of the medium after which the radioactivity of this fraction continued to increase for up to 4 hours. Little or no labeled lipid was found in the $D > 1.21$ fraction, indicating that the lipoproteins were effectively concentrated into the upper 2.5 ml of the tube by the flotation technique used.

The time course for appearance of labeled lipid in the tissue lipoproteins ($D < 1.21$ fraction) is shown in Figure 3. The incorporation of lipid radioactivity into these intracellular lipoproteins showed less of an initial time lag but fell off more rapidly, perhaps because of exhaustion of the small amount of labeled acetate used. Evidently transfer of label to the medium continued to occur even though the incorporation of label into intracellular lipoproteins after 1.5 hours of incubation was minimal. It should be noted that the total amount of lipid radioactivity in the intracellular lipoproteins was 15 to 20 times greater than the amount appearing in the lipoproteins of the medium. Effective flotation of tissue lipoproteins was demonstrated by the minimal amounts of lipid radioactivity found in the middle and bottom layers of the tube (Figure 3).

The specific radioactivity of the protein moiety of the lipoproteins in the medium and in the liver slices is shown in Figure 4 as a function of time. After an initial lag period, similar to that seen for appearance of lipid radioactivity, the specific radioactivity of the lipoprotein-protein in the medium increased almost linearly with time for 4 hours. Incorporation into the protein of the slice lipoproteins began more rapidly, and the specific radioactivity of this fraction was higher than that of the lipoproteins in the medium except at the 4-hour point. The data are consistent with the expected precursor-product relation between the intracel-

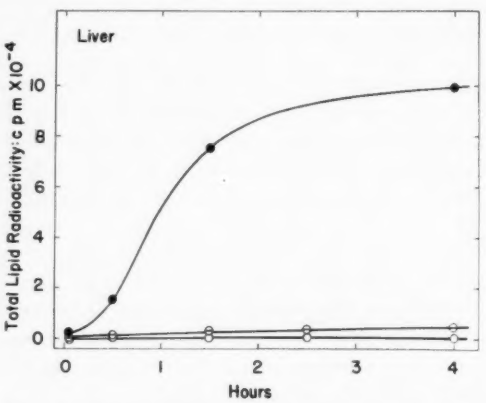

FIG. 3. TOTAL LIPID RADIOACTIVITY IN TISSUE SLICE LIPOPROTEINS ($D < 1.21$) AS A FUNCTION OF TIME OF INCUBATION. Rat liver slices were incubated in Krebs' bicarbonate buffer containing 1-C^{14}-acetate and 1-C^{14}-leucine. Results are shown for counts in the upper 2.5 ml (●——●), the middle 2.0 ml (○——○) and the bottom 8.5 ml (⊖——⊖) of the tube (key as in Figure 2).

lular lipoproteins and the lipoproteins of the medium.

Net changes in lipoprotein levels during incubation. The amounts of both protein and phospholipid in the lipoprotein fraction of the tissue slices increased during the first 1.5 hours of incubation but then showed no further increase at 4 hours. The amount of lipoprotein-protein increased from 470 μg per g of liver at zero time to 730 μg per g at 1.5 hours; the lipoprotein-phospholipid increased from 160 μg per g of liver at zero time to 230 μg per g at 1.5 hours. No attempts were made to characterize structurally the lipoproteins in the tissue slice. To what extent these represent newly synthesized plasma proteins and to what extent they represent exclusively intracellular lipoproteins is not known. Despite these measurable increases in tissue slice lipoprotein there was no demonstrable increase in the amount of lipoprotein in the medium. It should be noted that the concentration of lipoprotein in the medium was extremely low, about 1 μg per ml of medium. Losses due to denaturation or adsorption or losses during isolation cannot be ruled out. The fact that the *specific radioactivity* of the medium lipoprotein-proteins continued to rise linearly with no concomitant rise in their concentration might also be due to re-entry of medium lipoproteins into the slice or exchange of intracellular and extracellular lipoproteins.

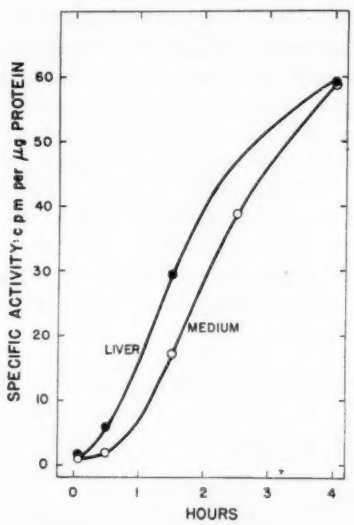

FIG. 4. SPECIFIC RADIOACTIVITY OF THE PROTEIN MOIETY OF THE D < 1.21 LIPOPROTEINS. In liver slices (●——●) and in medium (○——○) during incubation in Krebs' bicarbonate buffer containing 1-C^{14}-leucine and 1-C^{14}-acetate.

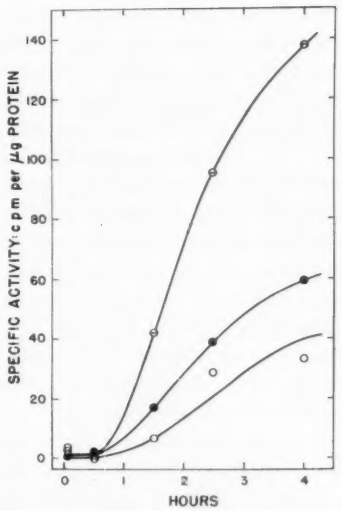

FIG. 5. SPECIFIC RADIOACTIVITY OF THE PROTEIN MOIETY OF THE MEDIUM PROTEINS IN THREE DIFFERENT LAYERS OF THE CENTRIFUGE TUBE AFTER CENTRIFUGATION AT DENSITY 1.21. Rat liver slices were incubated in Krebs' bicarbonate buffer containing 1-C^{14}-leucine and 1-C^{14}-acetate for the times indicated (key as in Figure 2).

Observations on the ultracentrifugal separation of lipoproteins from infranatant proteins. The flotation technique used in these studies for isolation of lipoproteins is essentially that described by Havel, Eder and Bragdon (22), a standard procedure that has been used by many other workers studying serum lipoproteins. That the lipoproteins are effectively concentrated into the upper 2.5 ml of the tube by this procedure is shown by the data of Figures 2 and 3 discussed above. The 2.0 ml of solution immediately below this top fraction, however, was found to contain protein. In other words, there was no protein-free zone clearly separating the lipoprotein fraction from the residual fraction. The concentration of protein in the middle layer was actually greater than that in the top layer. The extent to which the top layer was contaminated with what appear to be slowly sedimenting non-lipoprotein proteins could not be determined directly. However, the specific radioactivities of the proteins in top, middle and bot-

tom layers were separately measured, and the results are shown in Figure 5. In the incubation medium the bottom fraction was found to have the highest specific radioactivity, most likely due to newly synthesized and secreted serum albumin (23). The lipoprotein fraction at the top was next highest in specific radioactivity and the protein of the middle layer the lowest. Thus the top and bottom fractions were separated by a fraction of lower specific radioactivity, indicating that the lipoprotein fraction at the top was probably not contaminated by material of high specific radioactivity. No finer separation of the middle layer was attempted, and it may be that the protein of this fraction is indeed separated from the top layer by a narrow protein-free zone. The best evidence that this may be the case is the fact that the peptide patterns of the D 1.063–1.21 fraction from the medium, described above, and their autoradiograms coincide with the pattern of serum D 1.063–1.21 lipoproteins. On the other hand the data do not exclude the possibility that the gradient of sedimenting proteins may have extended up into the top 2.5 ml. These results and the results of Avigan, Eder and Steinberg (24) show that the routine methods used for preparative isolation of lipoproteins, while adequate for most purposes, must be carefully re-examined in each specific application and particularly in experiments involving measurements of lipoprotein-protein specific radioactivity. In the following experiments the layering technique and filtration step described under Methods were employed to minimize contamination by slowly sedimenting proteins or by particulate matter.

Relation between rates of cholesterol biosynthesis and rates of lipoprotein synthesis and secretion. Implicit in many discussions of cholesterol metabolism is the assumption that changes in the rate of cholesterol biosynthesis are reflected in changes in the rate of lipoprotein production and thus in serum lipoprotein levels. With the system described here it was possible to explore the question by inducing changes in the rate of cholesterol synthesis by appropriate pretreatment of the intact rat and then direct measurement of the rate of lipoprotein synthesis in liver slices incubated in serum *in vitro*. Stimulation of cholesterol synthesis in the liver was induced by intravenous injection of Triton WR-1339 (25). Inhibition of cholesterol synthesis was produced by feeding a diet containing 1 per cent cholesterol (26).

Liver slices from control rats and from rats pretreated as described were incubated in a serum medium to which were added both acetate-1-C^{14} and leucine-1-C^{14}, to permit simultaneous measurement of cholesterol synthesis and lipoprotein-protein synthesis. As shown in Table I, the rate of incorporation of radioactivity into total liver cholesterol was increased approximately 150 per cent by injection of Triton and decreased approximately 90 per cent by cholesterol feeding. Despite these large changes in the rates of cholesterol synthesis there were no significant changes in the rates of incorporation of radioactivity into the protein moiety of secreted lipoproteins. The rate of appearance of labeled cholesterol in the medium lipoproteins was not measured in these experiments, and no conclusions can be drawn regarding the influence of changes in biosynthetic rate on the rate of transfer of labeled sterol to the medium. The assumption is made that the rate of exchange would be similar in control and experimental liver slice incubations, since the medium contained equal concentrations of lipoprotein available for exchange reactions. In order to

TABLE I

Changes in rate of cholesterol synthesis without change in rate of lipoprotein-protein synthesis *

Group	No. of rats	Tissue non-saponifiable lipid radioactivity	Medium lipoprotein-protein radioactivity	Lipoprotein-protein radioactivity Residual protein radioactivity
		cpm X 10^{-3}/g liver		
Control	6	100 ± 16	6.9 ± 1.6	1.6 ± 0.18
Triton-treated	6	250 ± 82	8.4 ± 1.5	1.8 ± 0.34
Cholesterol-fed	6	11 ± 0.54	6.8 ± 1.1	2.1 ± 0.39

* Liver slices (500 mg) were in each case incubated for 2.5 hours at 37° C under 95% O_2:5% CO_2 in 5 ml of normal rat serum containing 10 μc sodium acetate-1-C^{14} and 15 μc DL-leucine-1-C^{14}. Nonsaponifiable lipids were isolated from the liver slices only; lipoproteins were isolated from the medium only, as described in the text.

check on the possibility that the pretreatment given the rats might have altered the general pattern of protein biosynthesis, and thus have masked an effect on lipoprotein biosynthesis, the specific radioactivity of the residual protein fraction was determined. For each group the specific radioactivity of the lipoprotein in the medium relative to the specific radioactivity of the residual protein fraction is shown in the last column of Table I. The differences are not statistically significant.

Comparison of rates of lipoprotein synthesis in normal and in nephrotic rats. Nephrotoxic serum nephritis was produced in a group of rats by injection of antikidney serum prepared in rabbits by the method of Baxter and Goodman (*7*). These animals develop marked proteinuria, hypoalbuminemia and strikingly elevated serum lipid levels. Serum lipid levels measured in two of the four rats studied were, respectively: total cholesterol, 640 and 336 mg per 100 ml; phospholipids, 632 and 386 mg per 100 ml; triglycerides, 3,150 and 1,380 mg per 100 ml. Liver slices from nephrotic rats and from normal rats were incubated with leucine-1-C^{14} in normal rat serum and the radioactivity appearing in the lipoprotein-proteins of the medium ($D < 1.21$ fraction) was measured. Results are expressed in terms of the specific radioactivity of the lipoprotein-proteins. Since, as shown in separate studies, the amount of lipoprotein in the serum medium does not change measurably during incubation these values are proportional to the *total amount* of radioactivity appearing in the medium lipoproteins. The specific radioactivity of the proteins in the residual fraction was also determined. As shown in Table II, the incorporation into both fractions was, with nephrotic rat liver slices, almost double the incorporation observed with normal rat liver slices. One possible interpretation of these results is that the true rate of synthesis of lipoproteins and also of the non-lipoprotein proteins, principally albumin (*23*), is increased in the nephrotic rat liver. An increased net synthesis of albumin by liver slices from nephrotic rats has been reported previously by Marsh and Drabkin (*27*). Overproduction of both would be quite compatible with the observed serum lipid concentrations, since the albumin is being lost at a rapid rate in the urine while the lipoproteins, being much larger, are not so lost. On the other hand there are no data to rule out differences in pool size of precursor amino acids or pool size of preformed, unlabeled intracellular proteins and lipoproteins.

TABLE II

Lipoprotein synthesis in liver slices from normal and nephrotic rats *

	Specific radioactivity of medium proteins	
	D <1.21 fraction	D >1.21 fraction
	cpm/mg	
Normal rats	760	300
	940	380
Mean	850	340
Nephrotic rats	1,100	550
	1,600	710
	1,600	570
	1,800	600
Mean	1,520	600

* Incubation and isolation procedures as described in footnote to Table I.

DISCUSSION

The present studies demonstrate that it is possible to explore the mechanism of lipoprotein biosynthesis and some of the factors controlling it by using an *in vitro* system. Little or nothing is known at present about the mechanisms by which the apparently quite specific lipid-protein complexes found in the serum are put together. The fact that virtually all of the lipid of serum lipoproteins can be stripped from the protein moiety by extraction with organic solvents indicates that the binding of lipid to protein is loose, largely the result of non-covalent linkages. It would be of great interest to explore the steps involved in the organization of the lipoprotein molecule, and the system described here may be valuable in such studies.

It has been unequivocally shown that the liver slice *in vitro* synthesizes and secretes high density lipoproteins (D 1.063–1.21) identical with those found in the serum. Thus it is established that the liver is a source, although not necessarily the only source, of the protein moiety as well as of the lipid found in this class of serum lipoproteins. The data with respect to low density lipoproteins, while suggestive, are inconclusive. Marsh and Whereat, on the other hand, have shown that at least some of the low density lipoproteins secreted

by rat liver slices are identical immunochemically with serum low density lipoproteins (*20, 21*).

Using the *in vitro* liver slice system it has been possible to study factors influencing rates of synthesis of lipoproteins. It was shown that the rate of synthesis of cholesterol can be varied over a wide range without altering the rate of synthesis of the protein moiety of the lipoproteins. Evidently the synthesis of new cholesterol is not rate-limiting in the production of lipoprotein molecules under these conditions. Studies by Haft and colleagues (*28*) on the incorporation of labeled lysine into lipoproteins by perfused rat liver have led to a similar conclusion. They found that depression of cholesterol synthesis by feeding of cholesterol or stimulation of cholesterol synthesis by pretreatment with X-rays did not cause parallel changes in synthesis and secretion of lipoprotein-protein into the perfusate. It should be noted, however, that these *in vitro* studies cover only a relatively short interval of time and that the preformed cholesterol available in the tissue may have been adequate to support lipoprotein production at a normal rate over this short interval of time. The possibility that alterations in the rate of cholesterol synthesis may ultimately alter the rate of lipoprotein production if studied over longer periods of time cannot be ruled out.

Liver slices taken from rats with the nephrotic syndrome incorporated labeled amino acids into lipoproteins more rapidly than did liver slices from normal rats. The rate of synthesis of the non-lipoprotein-proteins (residual proteins) was likewise increased. Marsh has recently reported that the *net* synthesis of low density lipoproteins by perfused livers from nephrotic rats is much greater than the rate seen with normal rat livers and that albumin synthesis is likewise increased, although to a lesser extent (*29*). The results are compatible with the hypothesis that the hyperlipoproteinemia of nephrosis is in part due to overproduction of lipoproteins.

SUMMARY

Rat liver slices incubated *in vitro* incorporated labeled amino acids into the protein moiety and labeled acetate into the lipid moiety of lipoproteins. The high density lipoproteins (D 1.063–1.21) secreted into the medium were shown to be identical with the normally circulating high density lipoproteins, according to the peptide-pattern or "fingerprint" method of Ingram. Depression of the rate of cholesterol synthesis by prefeeding of cholesterol or acceleration of the rate of cholesterol synthesis by pretreatment with Triton did not significantly alter the rate of incorporation of amino acids into lipoproteins. The rate of synthesis and secretion of the protein moiety of lipoproteins as well as other proteins was elevated in liver slices of nephrotic rats.

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THE INTRAHEPATIC CONJUGATION OF SULFOBROMOPHTHALEIN AND GLUTATHIONE IN THE DOG *

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In 1950 Brauer, Krebs and Pessotti (1) reported that S^{35}-labeled sodium phenoltetrabromophthalein disulfonate (more commonly known as sulfobromophthalein, Bromsulfalein or BSP) may be recovered from the bile of the dog, during constant intravenous infusion, in four fractions which are separable by column chromatography. Similar fractions, accounting for the bulk of BSP excreted and having absorption spectra like those of standard BSP, have also been detected in the bile of sheep, rat, cat and chicken and in bile produced by the isolated perfused liver of the rat (2). More recently, Meltzer, Wheeler and Cranston (3) have found that BSP in the blood and bile of the dog and man may also be fractionated by paper chromatography and that several of the fractions yield a positive ninhydrin reaction suggesting the presence of amino acid. Glutamic acid and glycine (but not cysteine) were identified by two-dimensional chromatography following 6 N hydrochloric acid hydrolysis of an eluate of the fractions. Hence they concluded that BSP is conjugated with amino acid, presumably in the liver, since the conjugates were found in the bile prior to their appearance in the blood. Subsequent work by Javitt, Wheeler, Baker and Ramos (4) has shown that cysteine is also present in the conjugates. Similar findings have been made independently by other workers (5–7) during the past year. Glycine and glutamic acid have been demonstrated in acid hydrolysates by Combes (5) and cysteine has been found in hydrobromic acid hydrolysates of BSP conjugates by Grodsky, Carbone and Fanska (6, 7). As both Combes and Grodsky have also noted, the appearance of glycine, glutamic acid, and cysteine together would strongly suggest that glutathione might be conjugated with BSP. This inference has been substantiated in the course of the studies to be presented below.

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METHODS

The biliary excretion of BSP was measured in 5 trained unanesthetized fasting dogs before and after the intravenous administration either of single doses ranging from 200 to 300 mg or of BSP infusions over a prolonged period, in most instances with the establishment of high plasma BSP concentrations. Bile was collected continuously by means of an indwelling catheter placed in the common bile duct under direct vision through a Thomas duodenal cannula (8, 9). Cholecystectomy and splenectomy were performed when the duodenal fistula was prepared and the cannula inserted, several months prior to study. All bile samples were refrigerated or frozen as soon as possible after collection.

Aliquots of whole bile were applied directly to Whatman no. 1, 3 or 3 MM filter paper for one-dimensional chromatography or electrophoresis. Preliminary removal of proteins and bile salts was necessary in those specimens to be hydrolyzed for amino acid analysis. For this purpose 2 ml aliquots of bile were dialyzed in Visking cellulose tubing for 24 hours against equal volumes of distilled water. The dialysates were lyophilized to dryness, extracted three times with absolute ethanol (10) and the residue reconstituted in distilled water.

Covered glass chambers were used for one-dimensional ascending and descending chromatography with butanol : acetic acid : water (4 : 1 : 2) as the solvent mixture. For paper electrophoresis (11), a Spinco series B Durrum type apparatus was used with a constant voltage power supply. Electrophoresis was carried out at 400 v for 4 hours or 120 v for 18 hours using 5 N acetic acid (pH 1.9) as the conducting medium. Two-dimensional ascending chromatography according to Dent (12) was carried out with phenol : water (4 : 1) followed by 2,4-collidine : 2,4,6-lutidine : water (1 : 1 : 1) as the solvent systems. Following electrophoresis or chromatography the papers were air-dried at room temperature.

For purposes of comparison, the chromatographic and electrophoretic migration of tetrabromphenolphthalein (Eastman Organic Chemical), tetrabromphenolphthalein disulfonate (BSP, Hynson, Westcott and Dunning), so-

dium taurocholate, and various pure amino acids (Nutritional Biochemicals Corp.), glutathione (Schwarz Laboratories), taurine (Eastman Organic Chemicals), cysteic acid, and purified BSP conjugates—dissolved in either distilled water or canine bile—were determined separately or in various combinations. The proteins present in bile were dyed on the paper with bromphenol blue (11). Amino acids, taurine, cysteic acid, and glutathione were detected by spraying with 0.1 per cent ninhydrin in water-saturated butanol or by dipping the paper into 0.1 per cent ninhydrin in acetone and heating at 100° C for 5 minutes. Bile salts were detected by Kritchevsky and Kirk's reagent (13) and tetrabromphenolphthalein, BSP, and BSP conjugates, by spraying with 15 per cent sodium carbonate or exposure to ammonia vapor. The BSP conjugates, as reported previously (3), were also found to develop color with ninhydrin. It was noted that BSP conjugates could be detected more readily at lower concentrations by the purple color appearing on alkalinization than by ninhydrin treatment. Hence, small but detectable amounts of the conjugates might be erroneously considered ninhydrin-negative.

Hydrolysis was carried out on the lyophilized dialyzed bile samples or on solutions of the various BSP conjugates prepared therefrom in the following manner. Preliminary electrophoresis was used to separate BSP and its conjugates as anions from the amino acids, pigments and other cations. The zone of the electrophoretic sheet bearing BSP conjugates (ninhydrin-positive) was cut out and eluted with water (adjusted to pH 6.0 to 6.5). The eluate was concentrated by lyophilization and applied to ascending or descending chromatograms from which the individual conjugates were cut out and eluted. Acid hydrolysis was performed in 6 N HCl at 100° C for 14 to 24 hours in sealed glass ampules. The hydrolysates were filtered, evaporated to dryness with gentle heating and air stream, and the last trace of HCl was removed *in vacuo* over KOH. Aliquots of the dried residue were dissolved in distilled water for two-dimensional chromatography. Alkaline hydrolysis was carried out in covered test tubes in 0.3 N NaOH at 60° C for 2 hours in a water bath. These hydrolysates were "desalted" electrolytically (Research Specialties Co., model 1930 A), and concentrated by evaporation prior to chromatography.

Reactive sulfhydryl groups before and after alkaline hydrolysis were detected by the sodium azide-iodine reaction described by Feigl (14). Neutralization of the alkalinized samples was necessary to prevent interference owing to formation of sodium iodide. Inorganic bromide was isolated from the synthetic reaction mixtures by paper chromatographic separation, described by Pollard and McOmie (15), using acetone: water (4:1) as a solvent. Bromide was precipitated on the paper by immersion in an ammoniacal (0.7 M NH_4OH) ethanolic (95 per cent) solution of silver nitrate (0.1 M) and "developed" by exposure to blue fluorescent light under standard conditions. The concentration could be determined quantitatively, by comparison with appropriate standard solutions of NaBr on the same chromatograms by using a Spinco analytrol. Over the range employed (0.015 to 0.03 M, 10 μl aliquots), concentration and densitometric readings showed a satisfactory linear correlation.

Glutathione in the conjugates isolated by electrophoresis and chromatography was determined by the method of Sachs and Brand (16) as glutamic acid. Since this method involves the formation of hydroxamic acid from a lactone ring, BSP itself might be expected to interfere. However, under the actual conditions employed (pH close to 7.0) it was found that BSP developed only a minimal amount of color compared with an equimolar concentration of glutathione. BSP could be recovered completely from the chromatograms by elution with a phosphate buffer solution (0.1 M, pH 10.4) and colorimetric analysis. Since glutathione did not interfere and since no change in optical density occurred in mixtures of glutathione and BSP during or after conjugation, conjugated BSP could be evaluated by reference to a standard BSP calibration curve. Glutathione labeled with S^{35} (specific activity 16.6 μc per mg) was obtained from Schwarz Laboratories. Radioautograms were obtained by placing Eastman Kodak No-screen X-ray film (in film holders) against the dried chromatogram or electrophoretogram and leaving it for 6 weeks prior to development.

RESULTS

Excretion of BSP and its conjugates in the bile of the dog. Regardless of the method of administration, biliary BSP always proved to be separable into several fractions by chromatographic analysis (Figure 1). All reacted with ninhydrin, with the exception of the fastest moving component (fraction I) which assumed the same chromatographic position as the commercially available or "standard" BSP. The ninhydrin-positive fractions varied considerably in number and concentration. As a rule, three major bands were distinguishable but, with improved chromatography, other components were detected. Fractions II and IV appeared to be homogeneous, whereas fraction III could be resolved into two additional components (a and b, Figure 1). On electrophoresis, also, biliary BSP could be separated into a number of fractions having differing mobilities (Figure 2). All moved toward the anode and the most rapidly moving component appeared again to be identical with standard BSP. When individual BSP conjugate fractions were eluted from the chromatograms and subjected to electrophoresis it was found that fractions II, IIIa and IV all migrated to the position represented by the darkest band in Figure 2. Fraction IIIb migrated in part to this position, but a major portion also appeared in the

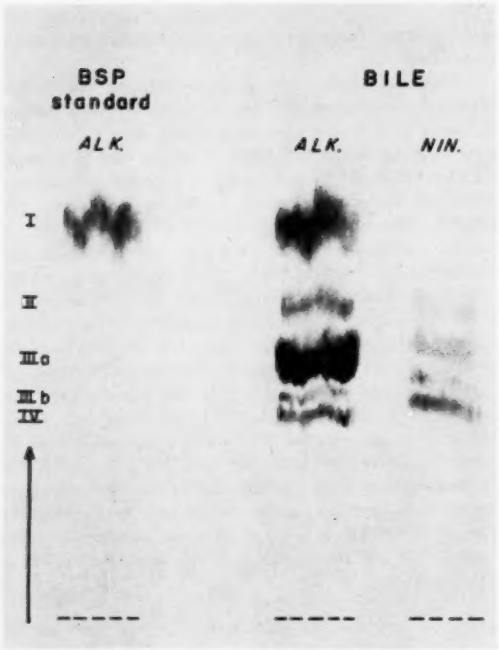


FIG. 1. CHROMATOGRAM OF SULFOBROMOPHTHALEIN (BSP) AND BSP CONJUGATES IN BILE OF THE DOG. Aliquots of solution of BSP (5 μg), BSP standard, and bile were applied at the points indicated by the broken lines. After alkalinization (Alk.) of the strips, standard BSP appeared as a single component whereas BSP appears in at least four additional fractions in bile—here labeled I, II, IIIa, IIIb, and IV for ease of reference. Fraction I corresponds to standard BSP, and does not give a positive ninhydrin reaction as do the remaining fractions (NIN). (These figures, and those that follow, have been prepared from tracings shaded in black to show relative intensities of colored bands more precisely than is possible with photographic reproduction.)

region marked by the upper ninhydrin band in Figure 2. The absence of significant hydrolysis under the conditions employed during electrophoresis was indicated by the fact that free amino acids could not be detected when purified conjugates were subjected to this procedure.

Analysis of the BSP conjugates. Acid hydrolysis of fraction IV yielded approximately equal amounts of glycine and glutamic acid. Although free BSP appeared in the reaction mixture, a large moiety of the BSP continued to migrate on chromatography as a ninhydrin-positive fraction. With alkaline hydrolysis also, large amounts of glycine and glutamic acid appeared, usually, however, in association with a small amount of a ninhydrin-positive material which behaved chromatographically like cysteine. Conversion of the relatively unstable cysteine to cysteic acid by the addition of hydrogen peroxide made it possible to demonstrate that cysteine was regularly present in alkaline hydrolysates in significant amount (Figure 3A). The azide-iodine test for reactive sulfhydryl groups, which was negative when performed on unhydrolyzed conjugates, became strongly positive after alkaline hydrolysis. These observations suggested that fraction IV is a complex of BSP and glutathione (glutamyl-cysteinyl-glycine) and that the linkage involves the sulfhydryl group of cysteine. The BSP-reacting ninhydrin-positive moiety observed after acid hy-

FIG. 2. ELECTROPHORETIC PATTERNS OF BSP AND BSP CONJUGATES IN BILE OF THE DOG. Standard BSP and its conjugates in the bile migrate toward the anode (+), whereas free amino acid (glutamic acid) that appears in the bile during the excretion of BSP moves toward the cathode (−) from the point of application (broken line).

drolysis of fraction IV might be a complex of BSP and cysteine.

Glycine and cysteic acid could be found following similar treatment of the chromatographically faster moving component of fraction III (Figure 3B). Glutamic acid appeared in trace amounts

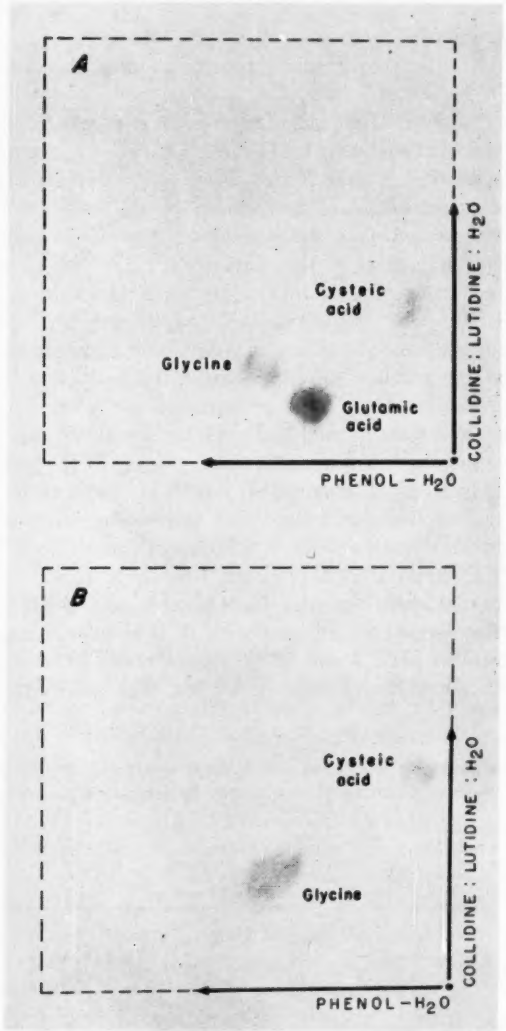


FIG. 3. CHROMATOGRAMS (TWO-DIMENSIONAL) OF ALKALINE HYDROLYSATES OF ISOLATED BSP CONJUGATES. A. Hydrolysate of fraction IV showing presence of glycine, glutamic acid, and cysteic acid following treatment with peroxide. B. Hydrolysate of fraction IIIa in which glycine and cysteic acid, but not glutamic acid, are detectable.

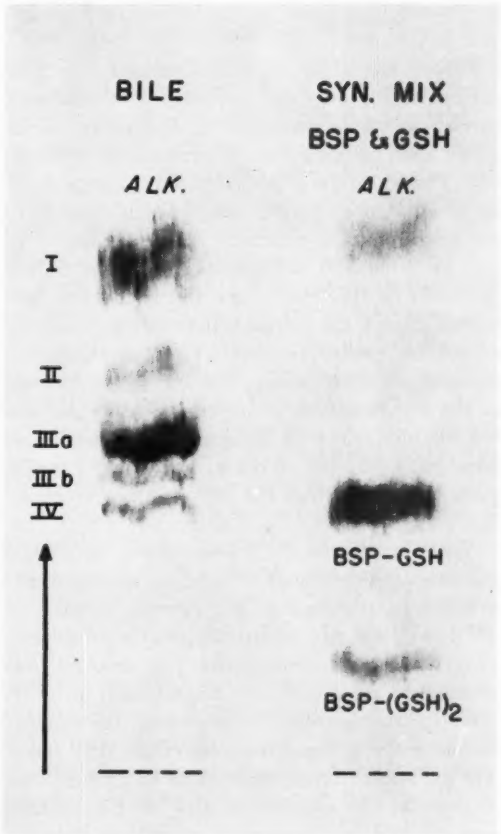


FIG. 4. CHROMATOGRAMS OF BSP AND BSP CONJUGATES IN BILE AND IN A MIXTURE OF BSP AND GLUTATHIONE. BSP and glutathione combined spontaneously in aqueous solution at 37° C to yield two detectable ninhydrin-positive conjugates. One having the chromatographic and electrophoretic properties of fraction IV appeared to be an equimolecular combination of BSP and reduced glutathione (GSH). The other, occasionally detectable in minute quantities in the bile, contained two molecules of glutathione for each molecule of BSP [BSP-$(GSH)_2$].

in early preparations but disappeared altogether as the separative procedure was improved. The more slowly moving moiety of fraction III which could be separated into two components by electrophoresis was not obtained in sufficient amount for complete analysis.

Fraction II behaved as a single substance both on chromatography and electrophoresis. Like the chromatographically faster moving portion of fraction III, it yielded glycine and cysteic acid with

appropriate treatment. Glutamic acid did not appear in the hydrolysates.

Protein-free (dialyzed) bile obtained from four normal fasting dogs not receiving BSP contained barely detectable quantities of free amino acids which were not significantly increased by hydrolysis. Eluates of the paper employed in these studies yielded on hydrolysis insignificant amounts of ninhydrin-positive material. An abundance of free glutamic acid did appear in the bile during excretion of BSP but, since the amino acid migrated toward the cathode, it could be easily removed by electrophoresis. Thus, amino acids, peptides, or other amino acid conjugates present in the bile, reagents or paper could not account for the abundance of glycine and glutamic acid which was observed in the hydrolysates of conjugates separated from the bile of dogs receiving BSP.

Synthesis of the BSP conjugates. BSP and glutathione were found to combine spontaneously *in vitro* in slightly alkaline aqueous solution at 37° C to yield two ninhydrin-positive conjugates (Figure 4). At the outset the mixture was brought to a pH of 7.3 by the addition of 0.1 N NaOH. As the reaction proceeded, the mixture became more acid and the color of the BSP faded. The pH was therefore maintained by dropwise admixture of the alkaline reagent over a 24-hour period. With exhaustion of glutathione by combination with BSP or by oxidation, the reaction came to a halt. The reaction was resumed if more glutathione was added. On both chromatography and electrophoresis two easily separable ninhydrin-positive BSP-reacting bands were demonstrable in addition to ninhydrin-negative standard BSP. Neither glycine nor glutamic acid was detectable. The major component appeared to be identical with fraction IV, chromatographically, electrophoretically and analytically. The second fraction seemed also to have its counterpart in a conjugate occasionally appearing in trace amounts in the bile of both the dog and rat.

Analysis of the eluted synthetic conjugates from glutathione and BSP indicates that the component apparently identical with fraction IV consists of an equimolecular combination of the two substances, whereas the second component (which is fluorescent in the ultraviolet) contains two molecules of glutathione for each molecule of BSP. During the reaction, hydrobromic acid is formed, accounting for the progressive acidification of the reaction mixture. Since the bromide released was found to be proportional to the amount of conjugate formed and since the conjugate contained no reactive sulfhydryl groups, BSP and reduced glutathione (GSH) probably combine by the formation of a thio-ether with replacement of bromine on the phthalic acid ring (Table I).

Cysteinylglycine prepared from pure GSH by partial hydrolysis with dilute phosphoric acid (17) also formed a conjugate with BSP *in vitro* when held at pH 7.4 and 37° C for 24 hours. Owing to the difficulty imposed by the high phosphate

TABLE I

Release of bromide during conjugation of sulfobromophthalein (BSP) and glutathione in vitro *

Exper. no.	Composition of synthetic mixture		Distribution of BSP in mixture after reaction			Amount NaOH added during reaction to keep pH constant	Computed hydrogen ion released	Observed bromide ion released
	BSP	GSH	Free	BSP-GSH	BSP-$(GSH)_2$			
	mmole			%			*mmole*	
I	0.30	0.30	52.1	43.4	4.5	0.13	0.16	0.15
II	0.42	0.42	44.4	51.4	4.2	0.21	0.25	0.24
III	0.14	0.25	22.7	68.0	9.3	0.10	0.12	0.12
IV	0.10	0.29	10.1	72.5	17.4	0.10	0.11	0.12

* In each experiment (except IV) aqueous solutions of BSP and reduced glutathione (GSH) containing the total amounts indicated under "Composition," were mixed and allowed to react at 37° C. The pH was raised to 7.3 and closely maintained by continuous titration with NaOH (0.1 N) in the total amount indicated in column 7. At equilibrium, 22.7 to 52.1% of the BSP remained unconjugated in Experiments I to III, 43.4 to 68.0% was conjugated as BSP-GSH and 4.2 to 9.3% as BSP-$(GSH)_2$. The amount of hydrogen ion which should have been released, computed as the sum of BSP-GSH and 2 × BSP-$(GSH)_2$, did not differ significantly from the amount of alkali actually added or the quantity of bromide ion released. In Experiment IV, 0.2 mmole of GSH was added to an aliquot of equilibrium mixture from Experiment II containing 0.1 mmole free BSP and 0.12 mmole conjugated BSP having the distribution shown for II. Under these circumstances the reaction came closer to completion and yielded a much larger quantity of BSP-$(GSH)_2$. Again the calculated value did not differ materially from observation.

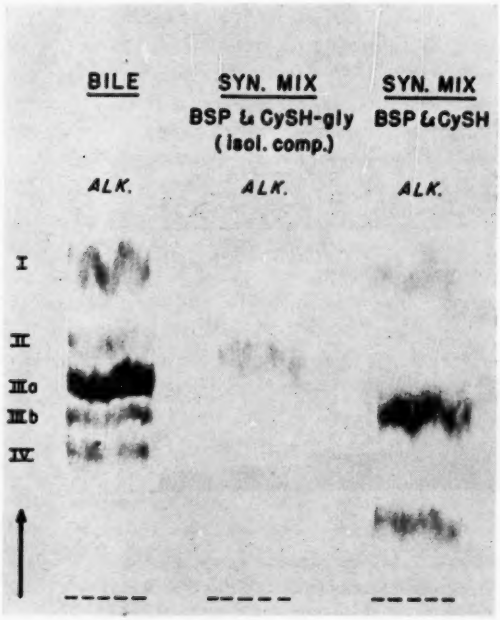


FIG. 5. CHROMATOGRAMS OF CONJUGATES PRODUCED IN AQUEOUS MIXTURES OF BSP AND CYSTEINYLGLYCINE OR CYSTEINE. Cysteinylglycine prepared from glutathione reacted with BSP to yield at least one conjugate, which behaved like fraction II when isolated from the synthetic mixture (Syn. Mix, center). At least two conjugates appeared in a mixture of BSP and cysteine, one of which resembled fraction IIIb (Syn. Mix, right).

concentration of this mixture, a preliminary chromatographic separation was necessary from which only the fastest moving portion was isolated. This material behaved electrophoretically, chromatographically (Figure 5) and analytically (two-dimensional chromatography after hydrolysis) like fraction II. Moreover, mild acid hydrolysis of fraction IV (or glutathione-BSP) resulted in the production of glutamic acid and fraction II. Thus, fraction II appears to be cysteinylglycine-BSP synthetically as well as analytically. Analysis of the faster component of fraction III indicates that it, too, is a cysteinylglycine-BSP. Cysteine and BSP have been found to combine *in vitro* to yield two conjugates of which the major seems to be identical chromatographically with the slower moiety of fraction III (Figure 5). On electrophoresis this conjugate moved to the position marked by the upper ninhydrin band in Figure 2.

Incorporation of glutathione sulfur in conjugates in vivo. Additional evidence that cysteine, derived from glutathione, appeared in the conjugates was adduced from the behavior of radioactive sulfur incorporated in glutathione during the excretion of BSP; 5 to 6 mg of glutathione labeled with S^{35} (50 to 100 μc) was injected intravenously 2 hours prior to administration of 300 mg of BSP. Bile samples were collected in the usual way and the radioactivity, in counts per minute per milliliter and BSP concentrations were determined in successive samples collected at 10- to 22-minute intervals. The biliary radioactivity and BSP concentrations rose, reached a peak, plateaued, and then fell simultaneously (Figure 6).

A one-dimensional ascending chromatogram was made from each sample and used for the preparation of a radioautograph by contact with X-ray film over a period of 6 weeks. The carbonate-sprayed chromatogram of two successive periods selected at the concentration peak, 40 minutes after the administration of BSP, and the radioautograph of the same patterns are illustrated in Figure 7. In every instance radioactive material was observed in the regions corresponding to BSP conjugates but not in the position occupied by unconjugated BSP. Since free glutathione was never detected in bile during any of the present studies, it is apparent that the S^{35} in these patterns must have been bound in the BSP conjugates.

It may be concluded, therefore, that sulfur derived from glutathione is a constant constituent of the BSP conjugates. It seems not unlikely that all of the conjugates have their origin in the combination of glutathione and BSP, fractionation resulting from the formation of isomers and from the cleavage of glutamic acid and glycine. The relatively small quantity of radioactive material detectable in the bile prior to administration of BSP was probably attributable to the excretion of breakdown products, since GSH did not appear in the bile in the absence of BSP.

DISCUSSION

The results of this study indicate that BSP and glutathione combine both *in vitro* and *in vivo* in the liver of the dog, presumably with the formation of a thio-ether linkage and release of bromine. In support of this view is the observation that a

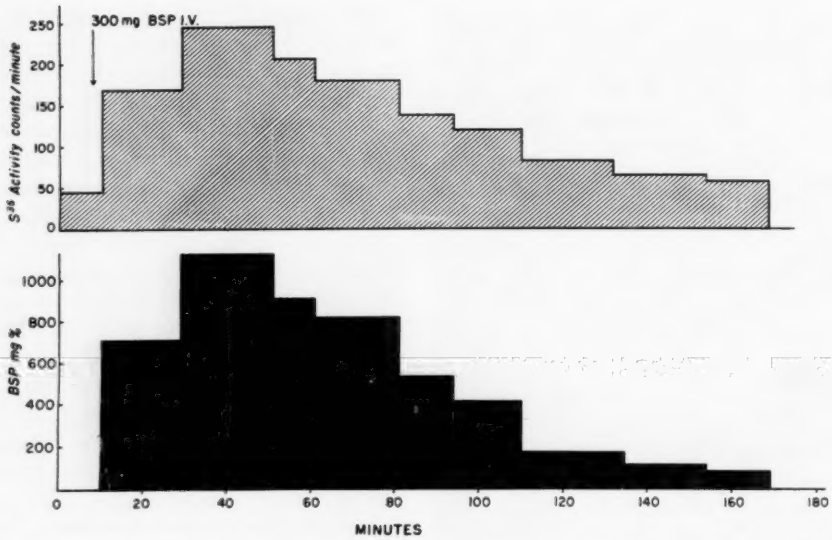
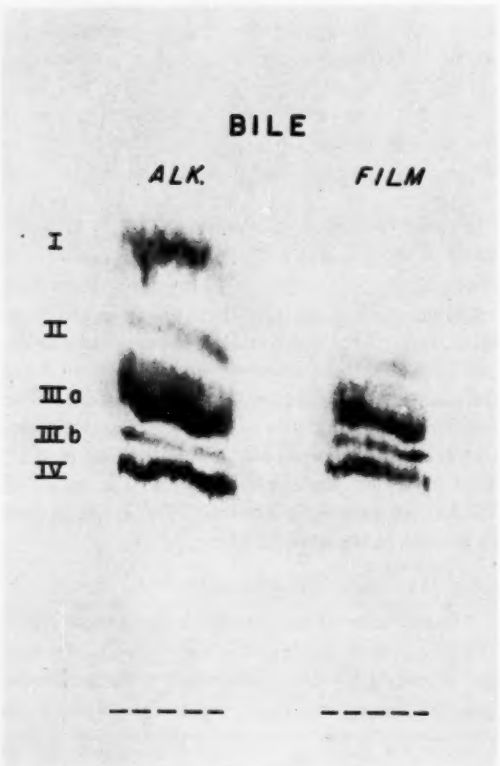


FIG. 6. BILIARY EXCRETION OF BSP AND RADIOACTIVE SULFUR IN THE DOG WHEN BSP WAS GIVEN 2 HOURS AFTER INTRAVENOUS ADMINISTRATION OF S^{35}-LABELED GLUTATHIONE. The biliary output of radioactive material increased markedly above the relatively low control value as BSP appeared in the bile. Biliary radioactivity and BSP concentration rose and fell together.



major BSP conjugate in dog bile (fraction IV in Figure 1), a ninhydrin-positive compound which yields glycine, cysteine, and glutamic acid on hydrolysis, can be synthesized by spontaneous combination of BSP with pure glutathione under conditions that preclude hydrolysis of GSH. Since an equimolar quantity of hydrogen bromide evolves during synthesis and since the conjugate possesses no free sulfhydryl groups, it may be inferred that combination involves linkage between sulfur and a carbon of the phthalic acid ring. A similar reaction has been described (18) as a means of hepatic detoxification of halogenated nitrobenzene and related compounds during the formation of mercapturic acid.

Cleavage products and isomers of a conjugate of BSP and GSH could account for all of the ninhydrin-positive, BSP-reacting substances appearing in the bile. The appearance of radioactive sulfur in all the biliary conjugates after administration of S^{35}-labeled glutathione clearly supports this conclusion. The presence of cysteinylglycine-

FIG. 7. RADIOACTIVE SULFUR IN BILIARY BSP CONJUGATES EXCRETED AFTER ADMINISTRATION OF S^{35}-LABELED GLUTATHIONE. Autoradiogram (film) of the alkalinized chromatogram of bile (Alk.); radioactivity is evident in every fraction except I (free BSP).

BSP conjugates, invariably in association with free glutamic acid, suggests that cleavage of glutamic acid may occur to some extent during excretion. The other possible products of cleavage, cysteinyl-BSP, glutamyl-cysteinyl-BSP and tribromo-BSP have not been identified. Isomerism could account for the chromatographic separation of two forms of cysteinylglycine BSP. Indeed, the appearance of a diglutathione conjugate of BSP in artificial synthetic mixtures attests to at least two sites on the BSP molecule at which combination may occur.

Regardless of the role of glutathione in BSP transport, whether essential or merely incidental, the loss of glutathione during BSP excretion could lead in principle to depletion of hepatic GSH. Such a depletion has actually been observed following the administration of a variety of substances that are excreted as mercapturic acids, but the dosages required have been much larger than those in which BSP has been administered in these or in the usual clinical procedures. Possibly a critical loss could be induced more readily in the liver, damaged by disease or hepatotoxic agents. These studies suggest that BSP may be used as a tool for the investigation of hepatic glutathione metabolism and its relation to hepatic disease.

SUMMARY

Sulfobromophthalein (BSP) and glutathione appear to combine intrahepatically in the dog to yield a variety of ninhydrin-positive conjugates. Since bromide and hydrogen ions are released stoichiometrically during synthesis of conjugates *in vitro*, it may be inferred that conjugation involves the formation of a thio-ether with replacement of bromine. Radioactive sulfur is found in all of the conjugates detectable in the bile when S^{35}-labeled glutathione is given with BSP, suggesting that all the ninhydrin-positive fractions may be derived from the conjugation of BSP and glutathione. This phenomenon may provide an approach to the quantitative investigation of hepatic glutathione metabolism in the experimental animal.

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STUDIES ON TRITIUM–LABELED DIGOXIN: TISSUE, BLOOD AND URINE DETERMINATIONS

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During the last 40 years extensive investigations have been reported on various aspects of the metabolism of cardiac glycosides. A detailed review describing the cellular basis of cardiac glycoside action has been reported by Hajdu and Leonard (1). One prime difficulty encountered in determining the metabolic fate of cardiac glycosides is in establishing a reliable and sensitive method for determining tissue, blood and urine levels of the glycosides as well as their derivatives in man and in experimental animals. The minute amounts present in blood, urine and tissues after oral or parenteral administration require extremely sensitive assay methods. Digitalis glycosides have been studied by utilizing bioassay (2–9), colorimetric (10–14), radioisotopic (15–17), and polarographic techniques (18, 19).

The behavior and fate of digitoxin in experimental animals and in man have been reviewed by Friedman, St. George and Bine (20). In Friedman's laboratory, bioassay techniques using embryonic duck hearts have detected 0.1 μg of digitoxin per ml of serum of humans who received 1.2 mg digitoxin intravenously (21). The method consists of the observation of cardiac irregularities in embryonic duck hearts immersed in test fluids (8, 22). This group has reported reproducible results in studies of experimental animals and humans (8, 20, 22–30).

Digitoxin labeled with carbon^{14} has been shown to be an excellent tool for appraising the behavior and fate of the glycoside (15–17, 31–38). Studies utilizing carbon^{14} digitoxin have included the fixation of cardioactive glycosides by isolated hearts (15); the tissue distribution and excretion in digitalis-sensitive and digitalis-resistant animals (16); the placental transfer of digitoxin in rats and guinea pigs (32); the renal excretion (33), blood levels (17), metabolic fate (34), and the placental transfer and fetal distribution (36) in humans.

The advantages of radioactive tracer methods for investigating the metabolic fate of digitoxin have been discussed by Sjoerdsma, Fischer and Johnson (15, 16), Okita and co-workers (17), and Geiling and Okita and co-workers (31–34, 36). The incorporation of a tracer substance with a higher maximal permissible radioactivity dose than that of carbon^{14} would make it possible to study various aspects of the metabolic pathways of the parent compound in greater detail. Thus it would be feasible to perform long-term studies in humans utilizing the radioactive glycoside exclusively. Data obtained from acute experiments carried out in laboratory animals might be correlated with results in humans. Recently, tritium-labeled digoxin (digoxin-H^3)[1] became available for experimental use. Extensive data on the utilization of tritium-labeled compounds were presented at three annual symposia in New York City (39, 40). The physiology and toxicology of tritium in man were studied extensively by Pinson and Langham (41).

This report presents preliminary data from studies in dogs using digoxin-H^3 administered intravenously.

EXPERIMENTAL METHOD

Unselected mongrel dogs weighing between 12 and 25 kg were anesthetized with Pentothal Sodium administered intravenously without prior medication. An endotracheal tube was inserted and respirations were assisted with a Phipps-Bird type respirator. Utilizing routine surgical techniques, polyethylene cannulas were inserted into the contralateral femoral artery and vein for the collection of blood samples. A superficial foreleg vein was cannulated for the administration of fluids and

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[1] Dr. Stanley T. Bloomfield, Burrough Wellcome, Ltd., kindly supplied the radioactive digoxin.

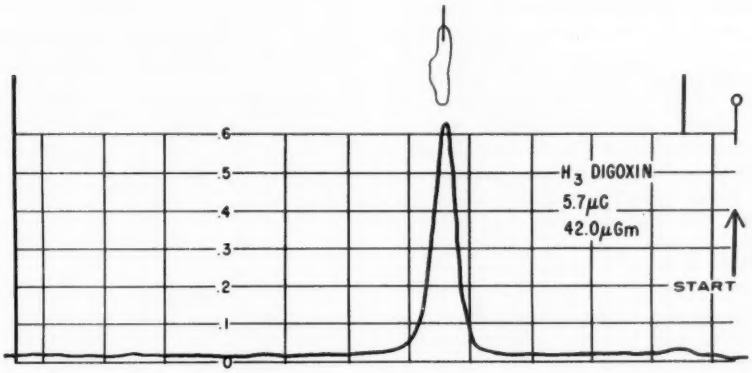

FIG. 1. COUNTING RECORD OF A CHROMATOGRAPHIC SPOT CONTAINING 42 μG OF DIGOXIN-H^3 (5.7 μC OF TRITIUM). Peak of radioactivity coincides with area of digoxin concentration.

drugs. The remaining superficial femoral artery was cannulated for recording intra-arterial blood pressures with a Statham pressure transducer. Needle electrodes were inserted and standard extremity electrocardiographic leads were recorded. Polyethylene catheters were introduced into the urinary bladder with 13 gage hypodermic needles or through a cystostomy wound. The right chest cavity was entered at the fourth intercostal space. The pericardium was incised and sutured to the chest wall. A purse string suture was placed on the right auricular appendage and a polyethylene catheter was guided into the coronary sinus. A transfixing suture was used to sustain the catheter in the coronary sinus with careful avoidance of blockage to the coronary sinus. The patency of the various catheters was maintained by a constant flow of heparinized saline. The arterial catheters were washed at repeated intervals with heparinized saline. Digoxin-H^3, and digoxin injection (Lanoxin) were mixed with 0.9 per cent saline in a syringe in the amounts specified in Table I. The tritium-labeled digoxin was made available in ampules containing 1 mg of digoxin labeled with 134 μc of tritium. The suspension was administered intravenously over one minute.

In order to make simultaneous collections, the serial collections from each blood vessel were made by three observers. Unless otherwise specified, samples were collected at 1, 3, 5, 10, 15, 30, 45 and 60 minutes after drug administration. Biopsy specimens were obtained from the right atrium and ventricle at predetermined intervals. Double mattress sutures were placed at the surgical site. At the termination of each experiment the animal was sacrificed. The liver, kidneys and heart were removed, weighed, and representative samples obtained. Total urine specimens were collected at various time intervals for measurement. The amount of fluid administered intravenously was determined for each study. Throughout the experiments, intra-arterial pressures and electrocardiograms were monitored and recorded.

Preparation of samples for determination of radioactivity

1. Blood. Ten ml of 95 per cent ethanol was added to 1 ml of heparinized blood in order to precipitate the protein. This was then centrifuged for 10 minutes at 2,500 rpm. Two ml of the clear supernate was removed and evaporated to dryness. The residue was dissolved in 0.4 ml of 95 per cent ethanol. Two-tenths ml was mixed with 15.0 ml of a solution made by mixing 0.01 g *bis*-phenyloxazolylbenzene (a wavelength shifter), 0.20 g 2,5 diphenyloxazole (a phosphor), and 100.0 ml toluene. The samples were transferred to special counting vials and cooled in a freezer at 0° C for at least 24 hours. All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer with a two-channel pulse-height analyzer. This instrument was calibrated daily, utilizing a standard containing 134 mμc of tritium-labeled digoxin. All samples were counted for 1 hour. Unless otherwise specified, counts were expressed in millimicrocuries per gram of tissue. Recovery experiments were performed by adding digoxin-H^3 to blood and extracting as indicated. Approximately 95 to 98 per cent of the added radioactivity was recovered by the procedure used.

2. Urine. The urine samples were diluted with 95 per cent ethanol to a final dilution of 1:2. Two ml was evaporated to dryness and the residue treated as described above.

3. Tissues. The tissue samples were dried with filter paper to remove blood and extracellular fluid. They were weighed, and preserved in 5.0 ml of 95 per cent ethanol until homogenized. Two ml of the homogenate was removed, evaporated to dryness, and treated as described above.

Chromatographic studies

Chromatographic studies were performed, using multiple solvent systems (see below) to identify the glycoside or its metabolites in urine and blood. These studies were necessary, since the concentration of radioactive material in blood and urine does not necessarily indicate

the presence of unaltered digoxin H^3. Control chromatographic observations were performed using alcoholic solutions of radioactive and nonradioactive digoxin, and mixtures of these solutions with blood and urine in multiple proportions (42). Urine samples were precipitated with 40 per cent lead acetate and the supernate was extracted three times with chloroform (3 parts chloroform to 1 part urine). The chloroform extract was evaporated to dryness, and the residue dissolved in a 1:1 chloroform: methanol solution. This final suspension was evaporated to a small volume and examined with chromatographic techniques. Details of these techniques are given by Gisvold and Wright (43).

Chromatographic studies of digoxin solutions were carried out utilizing the following solvent systems: 1) chloroform-benzene-formamide (44); 2) chloroform-water-methanol (44); 3) benzene-chloroform-butanol (45); 4) benzene-ethyl acetate-chloroform (46); 5) methyl-isobutylketone-isopropyl ether-formamide (43). Optimal results were obtained with a slight modification of system 5. The proportions by volume were: methyl-isobutylketone, 8; isopropyl ether, 2; butanol, 1; and formamide, q.s. to saturate.

The chromatographic strips were allowed to develop in a closed jar saturated with the solvent system described. They were then passed quickly through a solution of 2.5 per cent meta-dinitrobenzene in chloroform and sprayed with a solution of 5 per cent KOH in methanol. The strips were examined under white and ultraviolet light. The Rf values were calculated for each chromatogram. The spots were sectioned off the paper, eluted with isopropanol, and processed for radioactivity counting as described previously.

EXPERIMENTAL RESULTS

Blood levels of digoxin-H^3. Blood samples aspirated simultaneously from the femoral artery, femoral vein and coronary sinus showed a rapid decrease in radioactivity during the first 15 minutes (Table II).

Urine levels of digoxin-H^3. Significant levels of the radioactive isotope were measured in the urine samples collected within 30 minutes after the intravenous injection of tritium-labeled digoxin (Table III).

TABLE I

Digoxin dose schedule

Dog	Weight	Digoxin-H^3	Lanoxin	Vol. of injection
	kg	*mg*	*mg*	*ml*
1	25.0	0.5	0.125	5.0
2	12.7	1.0	0.5	10.0
3	13.6	1.0	1.0	10.0
4	18.1	1.0	1.0	10.0
5	10.9	2.0	0.5	10.0

TABLE II

Blood levels of digoxin-H^3

Dog	Time	Arterial	Venous	Coronary sinus
	min		*mμc/ml blood*	
1	1	184.14	49.84	117.56
	3	54.82	19.58	28.58
	5	34.90	13.34	21.40
	10	15.24	10.82	8.74
	23	8.88	7.34	4.60
	45	4.02	4.04	1.8
2	1	181.48	167.24	86.24
	3	81.56	75.38	96.94
	5	30.98	64.92	65.92
	10	26.92	41.64	43.06
	15	30.20	31.92	26.66
	30	15.44	14.32	14.46
	45	8.90	9.44	5.56
	60	10.60	7.80	10.26
	75	6.14	9.02	6.60
	90	4.46	4.64	4.20
3	1	238.30	153.40	158.20
	3	129.12	77.70	103.92
	5	112.02	60.16	79.94
	10	65.80	53.14	42.24
	15	26.80	34.26	38.50
	25	17.36	21.02	19.32
	35	12.32	17.14	13.26
	38	10.78	18.20	19.14
4	1	149.14	132.44	151.00
	3	69.26	80.16	105.46
	5	70.70	59.92	47.06
	10	47.58	47.38	36.86
	15	21.48	34.08	30.84
	30	26.82	27.72	15.04
	45	10.12	16.94	12.12
	60	12.44	12.48	12.70
5	1	324.00	119.58	199.82
	3	193.08	97.38	208.52
	5	90.24	214.32	142.18
	10	38.64	165.58	83.88
	15	20.38	66.78	29.90
	30	37.52	16.76	26.42
	45	8.70	19.98	32.58

Tissue levels of digoxin-H^3. Studies of the tissue levels of the isotope revealed significant amounts of radioactive digoxin in the liver, kidneys and heart. In tissue samples obtained at the end of the experiment, the highest concentration of radioactive digoxin per gram of tissue was found in the kidney. An average of 633.40 mμc was recovered per g of kidney. Studies of liver and heart revealed an average recovery of 128.48 and 72.35 mμc per g of tissue, respectively (Table IV). An average of 68.73 per cent of the total administered radioactive digoxin was recovered in the liver, heart and kidneys (Table V). These three organs accounted for an average of 4.1 per cent of the dog's body weight.

TABLE III

Recovery of digoxin-H^3 in urine

Dog	Time interval after i.v. injection of digoxin-H^3	Urine collected	Total recovered	Injected dose
	min	*ml*	*μc*	%
1	0–22	13	2.926	8.06
	22–45	16	2.476	
2	0–60	38	2.528	
	60–75	44	2.020	6.28
	75–85	65	2.870	
4	0–10	42	0.125	
	10–30	200	3.374	6.45
	30–45	100	5.152	

Chromatographic studies. Controlled chromatographic studies of digoxin solutions showed that the Rf values of the glycoside were not altered by chloroform extraction. Chromatographic spots, satisfactory for identification studies, have been obtained using as little as 0.006 mg digoxin in 25 μl of 95 per cent ethanol.

The chromatographic strips obtained by using radioactive samples were cut lengthwise and studied, utilizing a Chromatogram scanner (Baird Atomic) with rectilinear galvanometric recorder (Texas Instrument, Inc.). A representative "curve" is depicted in Figure 1. The spots were later sectioned off the paper, eluted and processed for counting as described previously (42).

DISCUSSION

A technique has been described which appears to be suitable for the appraisal of certain aspects of the metabolism of a cardiac glycoside in blood, urine and body tissues.

Forty minutes after the injection of tritium-labeled digoxin, approximately 68 per cent of the drug could be recovered from the heart, kidneys and liver. This percentage of recovery compares favorably with studies utilizing digitoxin-C^{14} (17, 33, 34). The relatively large amounts of the radioactive isotope recovered in the urine and kidneys corroborate data of other investigators who have shown that the major organ involved in the ultimate excretion of digitoxin is the kidney (34). Using the chromatographic techniques described, we have been able to identify the unaltered glycoside, digoxin, in dog urine in our acute, short-term experiments.

TABLE IV

Recovery of digoxin-H^3

Dog	Heart	Kidney	Liver
		mμc/g tissue	
1	47.00	147.48	14.26
2	80.30	899.32	48.40
3	113.62	525.70	124.54
4	64.30	388.34	82.80
5	156.51	1,206.18	372.44

Both digoxin and lanatoside C have been found to be excreted principally as the unchanged glysoside (38). A conjugate of digoxigenin has also been identified in rat urine after the administration of digoxin and lanatoside C (38, 47), and in rat bile after the administration of digoxin (48). Three separate bands have been obtained in chromatographic studies of rat urine after the administration of acetyldigitoxin (47). The band closest to the starting line could not be separated from metabolite B, a conjugate of digoxigenin.

TABLE V

Recovery of digoxin-H^3 from kidneys, liver and heart

Dog	Digoxin-H^3 injected i.v.	Digoxin-H^3 recovered	Digoxin-H^3 recovered
	μc	*μc*	%
1	67	40.886	61.02
2	134	97.628	72.10
3	134	98.680	73.64
4	134	78.572	58.63
5	268	209.753	78.26

After hydrolysis this product could not be separarated from digoxigenin. A second band which could not be separated from metabolite G, digoxin, was also hydrolyzed to digoxigenin. Further studies are being carried out in this laboratory to identify any metabolite that may be present in dog urine after the intravenous administration of radioactive digoxin. The nature of the radioactive material present in the kidney, heart and liver is being investigated in this laboratory.

From the data presented, it is evident that measurable levels of radioactive digoxin can be determined in blood, urine, heart, kidneys and liver of dogs receiving 3 to 7 μc of tritium per kg. According to published reports (49) "the maximal permissible amount of the radioisotope in the total body is 2×10^5 μc." Extrapolation of the data presented in this report suggests that satisfactory

levels of the isotope should be obtained in humans using digoxin-H^3 in dosages well within the permissible radioactivity. Preliminary observations regarded as satisfactory have been obtained in studies of urine and blood samples in one human (42). Apparently, studies of the metabolic pathways of digoxin in humans over long periods of time may be performed without dangerous exposure to radiation (50).

SUMMARY

Blood, urine and body tissue levels of tritium-labeled digoxin were studied in five mongrel dogs. The isotopic concentrations were measured by the liquid scintillation technique. Utilizing chromatographic techniques, the radioactive material excreted in the urine after the intravenous administration of the drug could not be distinguished from unaltered digoxin.

ACKNOWLEDGMENT

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THE *IN VIVO* GLUCURONIDE CONJUGATION OF RADIOACTIVE ETIOCHOLANOLONE AND ANDROSTERONE BY THE DOG KIDNEY *

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It is recognized that the liver readily conjugates reduced steroids (1) so rapidly that, under normal conditions, reduced steroids are not recovered in an unconjugated form in plasma but rather occur as sulfate (2–4), glucuronide (5), and probably phosphate (6) conjugates. Although Weichselbaum and Margraf reported the presence of small quantities of unconjugated tetrahydrocortisone [1] in 10 L of normal pooled plasma (7), confirming a previous observation in plasma from patients with advanced cirrhosis (8), this has not been the experience of others analyzing 50 to 100 ml of normal plasma. Moreover, most investigators have been unable to isolate significant amounts of unconjugated reduced androgen metabolites, viz., androsterone and etiocholanolone, in normal plasma [2] (2, 3, 9).

Recently, however, Bondy, Cohn, Herrmann and Crispell (10) demonstrated unconjugated etiocholanolone in the plasma of two patients with symptomatic etiocholanolone fever.[3] Conjugated etiocholanolone (12 to 15 mg per day) was present in the urine during the same symptomatic episode. When the patients were afebrile and without symptoms, the 24-hour urinary 17-ketosteroid patterns were normal, and unconjugated etiocholanolone was not detected in the plasma. It was postulated that an attack of this type of fever might be associated with *1*) transient overproduction of etiocholanolone and/or *2*) an intermittent inability to conjugate. The presence of conjugated etiocholanolone in urine and its absence in plasma during an attack of periodic fever is difficult to reconcile unless another organ in addition to the liver is involved in conjugation.

The rapid clearance of etiocholanolone and androsterone, after intravenous injection (12) and oral and intravenous administration of testosterone (13–15), suggests the possibility that these compounds may be conjugated by another organ besides the liver. The reports of Dutton (16), and Lipschitz and Bueding (17), that kidney slices synthesize glucuronide conjugates *in vitro* stimulated an investigation to determine whether dog kidney could conjugate radioactive etiocholanolone and androsterone *in vivo*.

MATERIALS AND METHODS

Six apparently healthy mongrel dogs (4 males and 2 females), weighing 8 to 17 kg, were used in these experiments. The dogs were anesthetized with Nembutal, 30 mg per kg. In order to insure an adequate diuresis, 5 per cent mannitol in hypertonic saline (166 mEq per L) was infused at a rate of 1.5 ml per minute through a cannula placed into the superior vena cava via the internal jugular vein. Through a mid-line incision the left kidney pedicle was exposed, the left ureter cannulated with polyethylene tubing (PE 205, Clay-Adams, New York, N. Y.) and the urine collected. After the renal vein was dissected free from the renal pedicle to the inferior vena cava, a polyethylene tubing (PE 160) with side holes cut near the tip was inserted into the renal vein at its junction with the spermatic (or ovarian) vein. At intervals this cannula was flushed with 10 ml

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[1] Compounds referred to are etiocholanolone (3α-hydroxy-etiocholane-17-one); androsterone (3α-hydroxy-androstane-17-one); tetrahydrocortisol (3α, 11β, 17α, 21-tetrahydroxy-pregnane-20-one); tetrahydrocortisone (3α, 17α, 21-trihydroxy-pregnane-11, 20-dione); allo-tetrahydrocortisol (3α, 11β, 17α, 21-tetrahydroxy-allopregnane-20-one); allo-tetrahydrocortisone (3α, 17α, 21-trihydroxy-allopregnane-11, 20-dione).

[2] Dubrulle and Claeys (9) measured unconjugated plasma 17-ketosteroids in 16 normal adolescents and found 7 to 28 μg per 100 ml.

[3] Recently, this clinical entity was named etiocholanolone fever in order to differentiate it from the familial Mediterranean fever on one hand, and the many periodic fevers on the other (11).

volumes of heparin-isotonic saline solution (0.2 mg per ml). The renal artery was dissected free and a ligature passed beneath it. At zero time the renal vein was cross-clamped at its junction with the inferior vena cava with a Potts bulldog (serrefine) clamp. Renal vein blood collection was begun and was manually controlled to prevent collapse or over-distention of the renal vein. Heparin-rinsed syringes were used for the blood collection. Systemic venous blood was collected via the jugular catheter during the same period and urine flow was also collected as a separate fraction. With the renal artery delivered up into the wound by traction on the ligature passed around it, thus serving to partially occlude the artery during the injection period, isotopically labeled steroid(s) (Table I), dissolved in 0.2 ml absolute ethanol and diluted to 2.0 ml with heparin-isotonic saline; were injected by direct needle puncture of the renal artery. The injection time varied from 15 to 30 seconds. It should be pointed out that the tip of the needle was positioned as closely as possible to the entrance of the renal artery into the kidney (Figure 1). In one experiment, a serrefine clamp instead of the sling ligature was used to constrict the renal artery at its origin. In order to eliminate any return of injected isotope by venous radicles in the renal capsule or by the lymphatics, the entire kidney as well as its pedicle was dissected from its bed and surrounded by a plastic envelope.

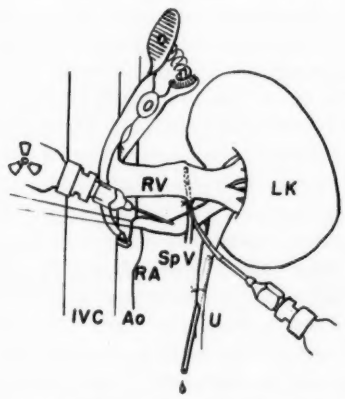

FIG. 1. A DIAGRAMMATIC REPRESENTATION OF THE DOG KIDNEY PREPARATION. The left ureter (U) is shown with the catheter in place. The spermatic vein (Sp V) is cannulated and a polyethylene catheter, with a side hole at its tip, is passed into the renal vein (RV). The renal vein is occluded with a serrefine bulldog clamp at its junction with the inferior vena cava (IVC) just as blood is withdrawn from the renal vein catheter. The rate of withdrawal is adjusted to prevent collapse or overdistention of the vein. As soon as the renal vein is occluded, isotopically labeled steroid(s) is injected rapidly by direct puncture of the renal artery (RA). A single ligature around the renal artery near its origin from the aorta (Ao) delivers the vessel into the wound and partially occludes it.

After injection of the isotope(s), full perfusion of the kidney was re-established by removal of the sling or the clamp. Up to 225 to 260 ml of renal vein blood was collected in 6 to 8 minutes. Hypotension occurred in only one dog. At the end of this collection time the pedicle was clamped and the renal pelvis washed three times with isotonic saline. These washings were added to the 4 to 6 ml of urine formed during the experimental period. Nephrectomy was performed at the conclusion of the experiment.

The plasma was separated from the cells by centrifugation within 15 minutes of collection (18) and frozen until analyzed. The urine sample and kidney were processed on the day of the experiment. The plastic bag and lymph contained in it, as well as the catheters, were analyzed for radioactivity.

Separation of plasma steroids

Free steroids. The plasma was thawed at room temperature. Two ml of 1 N NaOH per 40 ml of plasma was added, mixed, and the plasma extracted three times with 2 vol of freshly distilled chloroform. Emulsions were broken by centrifugation. The chloroform extract was dried in a rotating vacuum evaporator at 37° C and the residue dissolved in 10 to 15 ml of chloroform. Radioactivity (cpm) was determined with corrections for quenching. This extract (i.e., that portion of steroids extractable with chloroform without hydrolytic procedures) has been designated as the "free" (unconjugated) fraction. The redissolved residue was concentrated to dryness *in vacuo*, carefully dissolved in 1.0 ml of chloroform and applied as a 2.0 cm streak, along with appropriate reference standards, to washed (19) Whatman no. 1 filter paper in the heptane-propylene glycol system [4] (20). The chromatogram was scanned for radioactivity and the data from a count rate meter traced on an Esterline-Angus recorder. The pilot strip containing the reference standards and a 0.25 cm portion from the center of the "extract strip" were cut out and stained with Zimmermann reagent. There was no Zimmermann positive material detected on the "extract strip" except in the carbon^{14} experiment. However, there was very close agreement of R_f between the Zimmermann positive reference compounds and the location of the radioactive compound(s) by isotopic scanning. The appropriate area was cut out and eluted with 4.0 ml of absolute ethanol. An aliquot of this eluate was counted for radioactivity. The remainder was dried *in vacuo*, dissolved in chloroform and chromatographed in the 96 per cent methanol : heptane (1 : 1) system of Bush and Willoughby (21). The "free" compound(s) was located and counted as described.

Radioactivity was counted to a standard error of ± 2 per cent in a liquid-phosphor scintillation counter (Technical Measurements Co., model LP-2, New Haven, Conn.) which had a counting efficiency of 15 per cent for tritium and 65 per cent for carbon^{14}. When the

[4] All chromatograms were run in a room with constant temperature and humidity.

carbon scintillation counting eluates were low, they were quantitatively transferred to 2.0 sq cm areas on aluminum planchets at infinite thinness and counted in a specially constructed windowless gas-flow counter [5] which had a background of 1.5 to 2.0 cpm.

Conjugated steroids. After the chloroform extraction 2 volumes of absolute ethanol was added to the plasma. The precipitated protein was removed by centrifugation and further extracted three times with 1 volume of redistilled *n*-butanol saturated with water. The precipitate was discarded and the ethanol and butanol extracts were pooled and dried *in vacuo*. The residue was dissolved in 25 ml of 70 per cent methanol and washed three times with equal volumes of redistilled hexane. The hexane layers, which contained no significant radioactivity, were discarded and the washed methanol layer was taken to dryness *in vacuo*. The residue was redissolved in 1.0 ml of 70 per cent methanol. An aliquot was counted and the remainder streaked on Whatman no. 3 MM filter paper for electrophoresis. Reference standards of androsterone and etiocholanolone glucuronides and sulfates were applied adjacent to the streak. Paper electrophoresis was carried out as previously described (5). The conjugated compounds were located on the electropherogram by radioactive scanning and staining of the reference compound with Zimmermann reagent. The mobility of the Zimmermann positive standard coincided with the location of the radioactive peak.[6] No "free" androsterone or etiocholanolone was detected at the origin of the electropherogram by these techniques or after elution and counting (as described for tritium or carbon[14]). The sulfate area and remainder of the paper contained no measurable radioactivity after elution with 70 per cent methanol.

The radioactive conjugate area was cut out and eluted with 70 per cent methanol. An aliquot was counted and the remainder, along with adjacent reference standards, was chromatographed in the ethyl acetate : hexane : glacial acetic acid : water (120 : 80 : 60 : 140) system of Schneider and Lewhart (22). The steroid conjugate was located by radioactive scanning and staining with Zimmermann reagent, eluted, counted, and then rechromatographed in the *n*-butanol : butyl acetate : 10 per cent acetic acid (20 : 80 : 100) system (23). After radioactivity was measured, the eluate from the second chromatogram was hydrolyzed with β-glucuronidase [7] as previously described (5). The released "free" radioactive steroid was extracted with chloroform, counted, and chromatographed in the 96 per cent methanol : heptane system. The "free" compound was located, eluted, and radioactivity was determined as described above.

Separation of urinary steroids and steroids in lymph and plastic bag

The urine, lymph and plastic bag were each extracted twice with 100 ml of chloroform for "free" steroids. This was followed by extraction twice with 100 ml of *n*-butanol for conjugated steroids. The extracts were counted with allowances for quenching but were not processed further.

Separation of steroids from the kidney

The kidney was cut into small pieces, homogenized with 2 vol of 0.25 M sucrose and extracted three times with 100 ml chloroform washes. The extract was filtered through washed glass wool, concentrated to about 2.0 ml *in vacuo* and radioactivity measured.

The homogenate was extracted twice with 200 ml of *n*-butanol for conjugated steroids. This extract was filtered, concentrated and counted as described. The "free" and conjugated fractions were not characterized further.

RESULTS

Renal vein plasma. The total radioactivities of the "free" and conjugated fractions are listed in Table I. Percentages are corrected to 100 ml of plasma. These values are lower than were the actual radioactive recoveries (except experiment VI) since more than 100 ml of plasma was obtained. The uncorrected total "free" and conjugated radioactive steroid recoveries in the renal vein plasma represent 30 to 56 per cent of the original amount of radioactivty (cpm) injected. When tracer quantities of etiocholanolone-H^3 were injected, almost twice as much conjugated tritium-labeled etiocholanolone as "free" was found. There was twice as much "free" steroid as the conjugated form when tracer amounts of androsterone-H^3 were injected. After tracer amounts of androsterone-H^3 and etiocholanolone-H^3 were injected simultaneously, a greater amount of etiocholanolone glucuronide (16 per cent) than androsterone glucuronide was found (12 per cent of injected dose per 100 ml).

Jugular vein plasma, urine, lymph, plastic bag and kidney. No significant radioactivity was found in either the "free" or conjugated urinary fractions, tubings, lymph or plastic bag. There was 0.08 to 0.4 per cent of the dose "free," and 0 to 0.1 per cent of the dose radioactive conjugated fractions per 100 ml in the internal jugular vein plasma. After homogenization and extraction of

[5] Dr. Melvin V. Simpson of the Department of Biochemistry, Yale University, generously allowed us to use this specially constructed counter.

[6] In experiment VI (Table I) the androsterone glucuronide and sulfate areas were also located directly on 0.25 cm of the extract strips.

[7] Beta-glucuronidase was obtained from Warner-Chilcott Laboratories, Warner-Lambert Pharmaceutical Co., New York, N. Y.

TABLE I

"Free" and conjugated steroids in renal vein plasma

Experiment	Steroid*	Specific activity	Amount administered†	Dose recovered		Volume renal vein plasma
				"Free" steroid	Conj. steroid	
		μc/μg	*cpm*	*%/100 ml renal vein plasma*		*ml*
I	Androsterone-4-C^{14}	0.0035	2.22×10^4	15.5	10.5	142
II	Etiocholanolone-H^3	5.2	4×10^5	8	16	125
III	Androsterone-H^3	2.6	5.7×10^5	28	14	115
IV	Etiocholanolone-H^3	5.2	6.2×10^5	15	3‡	120
	Androsterone-H^3	2.6	6.1×10^5	20		
V	Etiocholanolone-H^3	5.2	6.4×10^5	8	16.5	122
	Androsterone-H^3	2.6	6.15×10^5	34	12	
VI§	Androsterone-H^3	2.6	2.75×10^5	18.2	13.3	90

* Androsterone-4-C^{14} was prepared biosynthetically from testosterone-4-C^{14} (New England Nuclear Corp., Boston, Mass.). The compound was isolated by gradient elution chromatography (24) after enzymatic hydrolysis and extraction of urine. Its identity was verified by infrared analysis. Androsterone (mp 181 to 183° C) and etiocholanolone (mp 149 to 151° C; donated by Dr. Seymour Lieberman, Columbia University, New York, N. Y.), were exposed to tritium gas (Wilzbach procedure) by the New England Nuclear Corp. The tritium-labeled steroids were thoroughly washed, purified, identified and characterized to constant specific activity by paper and column chromatography. The details of the method will be reported in a separate publication.

† The dosage of steroid represents the difference between the amount administered and the quantity extractable from the syringe and needle after injection.

‡ This animal was profoundly hypotensive 4 minutes after the injection of steroid. Because of the low percentage, conjugated steroids were not further separated.

§ Carrier androsterone glucuronide and sulfate (500 μg of each) were added to the renal vein blood at the conclusion of this experiment.

the renal tissue, 36 per cent "free" and 0 to 1 per cent conjugated, of the injected isotopic dose, were detected. A typical isotope recovery analysis is summarized in Table II.

Identification of androsterone and etiocholanolone glucuronides. The isolated radioactive steroid conjugates were identified as glucuronides of androsterone and etiocholanolone by virtue of their mobilities on paper by electrophoresis followed by chromatography in two systems. After separation of the compounds by these techniques, β-glucuronidase hydrolysis resulted in the release of the "free" steroid which was identified by paper chromatography.[8] Significant radioactivity was detected throughout the isolation and identification procedures.

DISCUSSION

These studies demonstrate that the dog kidney formed carbon^{14} and tritium-labeled androsterone and etiocholanolone glucuronide *in vivo*. Although the glucuronide portion of the steroid conjugate was not identified, it is unlikely that any other conjugate would behave in the manner described. Because of their negative charge, glucuronides of either the C_{21} or C_{19} steroids migrate as a class and at a slower rate than do the sulfates toward the anode on paper electrophoresis (5). Thus it is impossible to separate the C_{21} and C_{19} glucuronides by this technique. However, using Schneider and Lewbart paper chromatographic systems (22, 23), labeled etiocholanolone and androsterone glucuronides were easily isolated.

An insignificant amount of radioactivity was found in the peripheral plasma, indicating that the *in vivo* technique excluded the liver (1, 25) and duodenum (26) from this conjugation. The effi-

[8] In experiment VI carrier androsterone glucuronide and sulfate (Table I) were added to the renal vein plasma. After processing and purification by the methods described, the androsterone glucuronide fraction maintained constant specific activity. There was no detectable radioactivity in the androsterone sulfate fraction.

TABLE II

Percent isotope recoveries after the administraion of androsterone-H^3 into the renal artery

	Per cent of total dose
Total renal vein plasma (per 100 ml)	31.5
Peripheral venous plasma (per 100 ml)	0.08
Kidney	37.0
Plastic bag and lymph	1.0
Polyethylene tubings	0
Urine	3.0
Total	72.58

ciency of the dog kidney glucuronide conjugation system was 3 to 16.5 per cent of the injected isotopic dose per 100 ml per 6 to 8 minutes (Table I).

Because there are conformational differences between androsterone and etiocholanolone at the third carbon atom, speculation arises concerning the relative ease of conjugation of these compounds. The α-hydroxyl group at the third carbon atom in the 5β compound, etiocholanolone, is *equatorial* to the A ring. On the other hand, the hydroxyl group on this carbon atom is *axial* to the A ring and more sterically hindered in the 5α compound, androsterone. Since it is known that an esterifying reaction proceeds more rapidly when the acceptor group is *equatorial* rather than *axial* (*27*, *28*), it is reasonable to predict that under similar conditions more etiocholanolone than androsterone will be conjugated. Of course, this will be true only if the enzyme systems, cofactors, substrates and so forth are available to effect glucuronide synthesis.

The data in Table I favor this hypothesis since, under similar experimental conditions, a higher percentage of etiocholanolone glucuronide than of androsterone glucuronide was found. Slaunwhite and Sandberg (12) injected carbon^{14}-labeled androsterone and etiocholanolone into three women and found that the etiocholanolone glucuronides were cleared more rapidly than were the glucuronides of androsterone. Two of the three women had slightly higher levels of etiocholanolone glucuronides than of androsterone glucuronides, 15 minutes after injection. Thus, the more rapid conjugation of etiocholanolone as compared with androsterone may be explained by the conformational differences between the 3α-hydroxy groups of the two compounds. It follows, if this hypothesis is correct, that under similar conditions tetrahydrocortisol and tetrahydrocortisone may be conjugated more rapidly than are their allo derivatives, since the 3α-hydroxy group is *equatorial* to the A ring in the former, and *axial* in the latter compounds.

After conjugation, C_{19} glucuronides and sulfates are rapidly excreted by the kidneys (12–15). However, the actual physiological mechanism is not completely resolved. Bongiovanni and Eberlein demonstrated in a human volunteer that androsterone glucuronide was cleared from the kidney by tubular secretion (*29*). Kellie and Smith (30), on the other hand, reported clearance values for androsterone and etiocholanolone glucuronides comparable with those of inulin and suggested that these compounds may be excreted by glomerular filtration alone. In our experiments we were able to detect no more than 3 per cent of the injected radioactivity in the urine despite the large amount of free isotope "trapped" in the kidney. The significance of these findings is not understood and warrants further investigation.

Although it was demonstrated that the dog kidney synthesized steroid glucuronides *in vivo*, the site of renal conjugation and the eluicidation of this process in man remain to be determined.

SUMMARY

1. The conjugation of radioactive etiocholanolone and androsterone to their respective glucuronides was demonstrated (*in vivo*) in a dog kidney preparation. These glucuronides, isolated from the renal vein plasma, were characterized by their mobilities on paper electrophoresis, paper chromatography, hydrolysis with β-glucuronidase, and paper chromatography of the resultant "free" compound. Significant radioactivity persisted throughout the isolation and identification procedures. In one experiment androsterone glucuronide was characterized by purification to constant specific activity. No significant amounts of radioactive glucuronide conjugates were found in the urine or internal jugular vein plasma. Highly significant amounts of free isotope were "trapped" in the kidney parenchyma.

2. Three to 16.5 per cent of the injected dose of the labeled steroid per 100 ml of plasma was converted to the steroid glucuronide during the 6- to 8-minute perfusion of the kidney.

3. A hypothesis is presented which may explain the higher yields of etiocholanolone glucuronide than of androsterone glucuronide formed by this technique. The lesser steric hindrance of the *equatorial* α-hydroxyl group on the third carbon atom of etiocholanolone may facilitate rapid glucuronide conjugation. Although androsterone is conjugated readily, the rate of the reaction may be slightly less because of steric hindrance of the *axially* placed α-hydroxyl group on the third carbon atom.

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RATES OF I^{131}-LABELED THYROXINE METABOLISM IN EUTHYROID CHILDREN

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Studies of the requirements for thyroid hormone by infants and children suggest that they may be relatively higher than those of adults (1–3). Although the plasma concentration of thyroid hormone in newborn infants is appreciably higher than in adults, in children there appears to be no significant difference (4–9). The present study of thyroxine metabolism was designed to further define thyroid hormone metabolism in children.

MATERIAL AND METHODS

Seventeen children were included in this study; their ages varied between 3 and 9 years. All were institutionalized because of mental retardation[1] but showed no evidence of metabolic, endocrine or physical defects. They were all clinically and biochemically euthyroid, and maintained a height:age and osseous development corresponding to their chronological ages. Thyroxine labeled with radioiodine[2] was given intravenously. Beginning one day prior to injection, methimazole (Tapazole) in 10 mg doses every 8 hours was administered orally to each child for 10 days, to inhibit uptake of radioiodine by the thyroid. Serum protein-bound iodine (PBI), determined by the alkaline ash method of Barker, Humphrey and Soley (10), was estimated before and on the eighth day of the study. After the administration of radioactive thyroxine, heparinized venous blood samples were collected at 10 and 30 minutes at 1, 1.5, 2, 4, 8, 12 and 24 hours, and at daily intervals thereafter for 8 to 10 days. The heparinized plasma was separated promptly by centrifugation; 1 ml plasma samples were counted in a well-type scintillation counter in series with a standard of the same volume consisting of a suitable dilution of the injection material.

Urine and stool collections were made on two subjects. Aliquots of urine were counted in the same way as the plasma. The stools were collected in disposable tin cans, homogenized with water containing a few pellets of sodium hydroxide, and counted in series with a standard of equal volume over a wide well-type scintillation counter.

The radioactivity of each sample counted was expressed as a fraction of the injected dose.

RESULTS

The data were plotted on semilogarithmic coordinates as shown in Figure 1. A good fit of the data could be obtained with a minimum of three exponential components. There was a very fast initial component which was assumed to represent a mixing phase of the radioactive hormone during the first 2 hours after its injection. This component was therefore neglected in the analysis. The remaining two components —a relatively fast one followed by a slow component—suggested a two-compartment system, as shown in Figure 2. The half-life for radioactive thyroxine was calculated only for the slow component $\left(T_{\frac{1}{2}} = \frac{0.693}{\alpha_2}\right)$.

The method of Berman and Schoenfeld (11) was employed for the solution of the two-compartment system shown in Figure 2. Compartment 1 was considered to represent the plasma. The specific activity in the plasma (X_1) may be expressed as a sum of two exponentials:

$$X_1 = X_{11}e^{-\alpha_1 t} + X_{12}e^{-\alpha_2 t} \qquad [1a]$$

where X_{11} and X_{12} are the intercepts for the two components at time (t) = 0.

Normalized to $X_1 = 1$ at $t = 0$, Equation 1a can be rewritten as:

$$q_1 = A_{11}e^{-\alpha_1 t} + A_{12}e^{-\alpha_2 t} \qquad [1b]$$

where q_1 is the total radioactivity of the plasma (compartment 1) at any time t; $A_{11} + A_{12} = 1$.

The general solution of the turnover rates (λ's) of any two-compartment system may be given in terms of the constants of Equation 1b

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[1] The patients were selected from the D. C. Training School, Laurel, Md.

[2] Supplied by Abbott Laboratories, Oak Ridge, Tenn. Radioactive thyroxine was kept cold and away from light. It was tested for radiochemical purity by paper chromatography, and it was purified, when necessary, to 90 per cent purity or better.

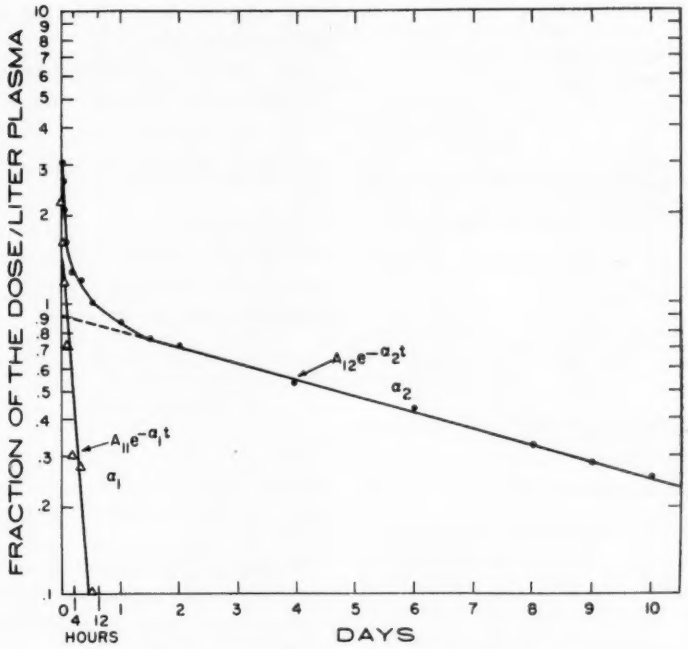

FIG. 1. SEMILOGARITHIMIC PLOT OF THE PLASMA DISAPPEARANCE CURVE FOR I^{131}-LABELED L-THYROXINE. α_1 and α_2 represent the exponential constants for the two components of the two-compartment system model.

as follows:

$$\lambda_{11} = \lambda_{01} + \lambda_{21} = A_{11}\alpha_1 + A_{12}\alpha_2 \quad [2a]$$

$$\lambda_{22} = \lambda_{02} + \lambda_{12} = A_{11}\alpha_2 + A_{12}\alpha_1 \quad [2b]$$

$$\lambda_{12} = \frac{A_{11}A_{12}}{A_{22}}(\alpha_1 - \alpha_2) \quad [2c]$$

$$\lambda_{21} = A_{22}(\alpha_1 - \alpha_2) \quad [2d]$$

$$\lambda_{01} = \lambda_{11} - A_{22}(\alpha_1 - \alpha_2) \quad [2e]$$

$$\lambda_{02} = \lambda_{22} - \lambda_{12} \quad [2f]$$

where A_{22} = the intercept for the radioactivity curve in compartment 2 which is unknown in this study; λ_{11} and λ_{22} = the total turnover rates from compartment 1 and compartment 2, respectively (Figure 2). To ensure that each of the λ's be positive, the range of A_{22} is limited by Equations 2a to 2f and must be as follows:

$$\frac{A_{11}A_{12}(\alpha_1 - \alpha_2)}{A_{11}\alpha_2 + A_{12}\alpha_1} \leqslant A_{22} \leqslant \frac{A_{11}\alpha_1 + A_{12}\alpha_2}{\alpha_1 - \alpha_2}. \quad [3]$$

Assuming that an amount Z of thyroxine per unit time enters the system through compartment 1, the volume of compartment 1 = (C_1) and the volume of compartment 2 = (C_2) may be obtained as follows:

$$C_1 = \left(\frac{A_{11}}{\alpha_1} + \frac{A_{12}}{\alpha_2}\right) Z \quad [4a]$$

$$C_2 = A_{22}\left(\frac{1}{\alpha_2} - \frac{1}{\alpha_1}\right) Z \quad [4b]$$

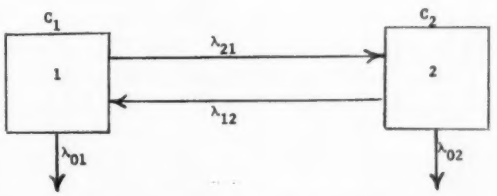

FIG. 2. A TWO-COMPARTMENT SYSTEM MODEL. C_1 = volume of compartment 1 and is equal to pool size of stable thyroxine with plasma as shown in Table I; C_2 = volume of compartment 2; λ_{01} and λ_{02} = the turnover rates from C_1 and C_2, respectively; λ_{21} and λ_{12} = the turnover rates from C_1 into C_2 and from C_2 into C_1, respectively; $\lambda_{11} = \lambda_{01} + \lambda_{21}$; $\lambda_{22} = \lambda_{02} + \lambda_{12}$.

from which

$$\frac{C_2 + C_1}{C_1} = 1 + \frac{A_{22}(\alpha_1 - \alpha_2)}{A_{11}\alpha_2 + A_{12}\alpha_1}$$

$$= 1 + \frac{\alpha_1 - \alpha_2}{\lambda_{22}} A_{22}. \quad [5]$$

Since the range of A_{22} is known from Equation 3, the range of $\frac{C_2 + C_1}{C_1}$ may thus be calculated.

The turnover rate of the system as a whole may be expressed as λ_{0T}:

$$\lambda_{0T} = \frac{\lambda_{01}C_1 + \lambda_{02}C_2}{C_1 + C_2} = \frac{\lambda_{01}}{\frac{C_1 + C_2}{C_1}} + \frac{\lambda_{02}}{\frac{C_1 + C_2}{C_2}}. \quad [6]$$

Again, using Equations 2, 3 and 5, the range of λ_{0T} may be calculated.

The values of all the λ's and the ratio $\frac{C_1 + C_2}{C_1}$ may not be uniquely determined without further assumptions (12). If one chooses to restrict the model of Figure 2 so that the degradation of thyroxine takes place in compartment 2 only, which presumably represents the extravascular space of thyroxine, λ_{01} equals 0; and Equations 2a and 2e yield

$$A_{22} = \frac{A_{11}\alpha_1 + A_{12}\alpha_2}{\alpha_1 - \alpha_2}. \quad [7]$$

This value of A_{22} permits the calculation of all the remaining λ's.

The volume of compartment 1 = (C_1) may be determined from the original data, since at t = 0,

$$(X_{11} + X_{12})C_1 = 1 \quad \text{or} \quad C_1 = \frac{1}{X_{11} + X_{12}}. \quad [8]$$

Assuming the plasma PBI is totally thyroxine, the effective extrathyroidal thyroxine (ETT_4) and the rate of degradation of thyroxine in micrograms per day (ρs/day) may be extimated:

$$ETT_4 = (C_1 + C_2) \cdot PBI \quad [9]$$

$$\rho s/day = (C_2) \cdot \lambda_{02} \cdot PBI \quad [10]$$

where ($C_1 + C_2$) = the effective extrathyroidal pool in liters; the PBI = micrograms per liter; and (C_2) = the volume of compartment 2 in liters.

In plotting the data on the urine and the stool, the rate of the appearance of the radioactivity in both was found to parallel that of its disappearance from the plasma. When expressed in terms of percentage of the dose, the radioactivity in the urine was about 4 per cent per 24 hours and that in the stool about 1 per cent per 24 hours of the remaining radioactivity in the body.

TABLE I

*Radioactive thyroxine metabolism in 17 children**

Subjects	Sex	Age		Weight	Surface area	PBI		Half-life	Turnover rate	Degradation rate	TVD	ETT_4	Plasma thyroxine
						Initial	8th day						
		yrs	*mos*	*kg*	m^2	*μg/%*		*days*	*% dose/day*	*μg/day*	*L*	*μg*	*μg*
L.A.	♂	5		17.5	0.7	5.9		4.9	13.7	24.2	2.986	177.1	55.1
J.A.	♂	5		15.5	0.6	6.2		4.3	15.5	16.8	1.745	108.9	39.4
E.D.	♂	3	11	15.8	0.6	5.9	6.6	5.8	11.7	15.6	2.133	133.7	40.4
T.L.E.	♂	4		15.0	0.6	5.2	6.2	4.7	14.3	14.0	1.711	97.4	34.1
R.J.	♂	3	5	15.0	0.5	6.7	5.6	4.4	15.0	25.6	3.152	195.4	46.5
L.B.	♀	5		16.2	0.6	7.1	7.4	5.4	12.5	19.9	2.167	156.9	53.4
R.G.	♀	4		14.8	0.6	7.1		4.6	14.4	21.2	2.079	147.5	49.9
D.M.	♂	6		18.2	0.7	5.2	6.2	3.9	16.8	27.8	2.953	165.3	58.0
K.M.	♂	6	6	17.8	0.7	5.7	6.5	5.5	12.8	19.3	2.567	158.7	54.0
W.T.	♂	3	9	14.2	0.6	7.0		5.1	13.2	23.7	2.531	179.7	55.4
W.T.	♂	3	11	14.5	0.6	7.1		5.1	13.4	13.8	1.488	102.9	31.6
R.K.	♂	8	9	16.9	0.7	5.1		5.9	12.3	15.8	2.721	139.0	60.3
M.L.	♂	8	7	16.7	0.7	6.9	5.7	5.3	10.5	19.2	2.881	182.9	51.9
D.W.	♂	3	4	12.9	0.5	5.2	6.2	4.5	18.7	20.0	1.891	106.8	33.7
S.L.	♀	4		13.6	0.6	7.3	6.6	4.2	16.0	21.5	1.913	134.2	51.6
L.B.	♂	5	5	15.8	0.6	4.4	4.7	5.1	13.1	23.7	3.964	181.0	36.8
D.B.	♂	3	7	11.4	0.5	5.1	4.9	5.5	12.3	12.5	2.024	101.6	32.7
Mean						6.06		4.95	13.89	19.68	2.406	145.2	46.16
SE						0.21		0.13	0.47	1.04	0.150	7.7	2.29

* PBI (initial, 8th day) indicates PBI values before and 8 days after Tapazole administration; TVD = effective total volume of distribution ($C_1 + C_2$) of thyroxine; ETT_4 = effective extrathyroidal thyroxine.

TABLE II

*Thyroxine metabolism in children as compared with reported values in adults**

	Child data		Adult mean	
	Range	Mean ± SE	Ref. 16 ± SE	Ref. 17
Half-life (days)	3.9–5.9	4.95 ± 0.13	6.7 ± 0.3	6.6
Turnover rate (% dose/day)	10.5–18.7	13.89 ± 0.47	10.5 ± 0.4	10.56
TVD (L)	1.488–3.964	2.406 ± 0.156	8.890 ± 0.624	9.400
(per m^2)	2.480–6.607	3.951 ± 0.252	4.559 ± 0.191	
(per kg)	0.103–0.210	0.156 ± 0.008	0.116 ± 0.005	
ETT_4 (μg)	101.6–195.4	145.2 ± 7.7	548 ± 38	508
(per m^2)	162.3–390.8	238.4 ± 13.1	281.8 ± 16	
(per kg)	6.5–13.0	8.9 ± 0.7	7.16 ± 0.4	
Degradation rate (μg/day)	12.5–27.8	19.68 ± 1.04	57.0 ± 4	53.6
(μg/m^2)	22.6–42.7	32.0 ± 1.61	29.4 ± 1.6	
(μg/kg)	0.9–1.7	1.29 ± 0.06	0.75 ± 0.04	

* See references 16 and 17; see footnote to Table I for abbreviations.

The radioactivity in the urine was assumed to represent the excretion of inorganic I^{131} which was liberated from the peripheral degradation of I^{131}-labeled thyroxine (13, 14). Fecal radioactivity, on the other hand, has been shown to be largely organic I^{131} (14), representing the end-product of the hepatic degradation of thyroxine [its conjugation and its hepatointestinal cycle (15)].

DISCUSSION

In general, the calculations described above give values for thyroxine turnover rate, half-time, degradation rate and extrathyroidal thyroxine pool (TVD) about 5 to 10 per cent higher than when the method of Sterling and Chodos (16) is used. The results of the present studies are listed in Table I, and in Table II they are compared with similar values obtained in adults by the Sterling-Chodos method and by others (16, 17).

Plasma thyroxine, when estimated empirically according to an estimated plasma volume for this age group of patients (18, 19), coincided with the calculated value of thyroxine in compartment 1. The ETT_4 or effective pool of thyroxine, on the other hand, seems to be about 30 per cent less than that of the extracellular fluid volume for this age group estimated by Edelman and colleagues (20). This apparent decrease in ETT_4 is possibly effected by the decreased thyroxine-binding capacity in the extracellular fluid as compared with that in the plasma (21), which occurs in a ratio of 1:2.

In Table II, the child seems to use thyroxine at a faster rate than does the adult, as suggested by the shortened half-life. His extrathyroidal thyroxine pool and thyroxine daily requirements are, however, about one-fourth and one-third those of the adult, respectively. In order to estimate the significance of this increase in the turnover rate of thyroxine in the child, the degradation rate and the extrathyroidal pool for thyroxine are calculated in terms of micrograms per square meter of surface area and micrograms per kilogram of body weight. When the surface area parameter is considered, the degradation rate of thyroxine in the child is not significantly larger than that in the adult. The effective extrathyroidal pool, however, is somewhat smaller in the child per square meter than in the adult. It would thus appear that the increase in thyroxine turnover in the child is more a function of the difference in body configuration than an actual increase in thyroxine utilization. When the body weight parameter is used, the conclusion favors an actual increase in thyroxine degradation by the child. It is quite possible that both factors play a role in the metabolism of thyroxine by the child, namely, a decreased pool and an increased turnover of thyroxine.

The children studied demonstrated no significant effect of age on either half-life or degradation rate for thyroxine. Nonetheless, since infants or adolescents were not included in this study, it is difficult to predict the variation that may exist among these three age groups, and in comparison with the adult.

SUMMARY

The metabolism of labeled thyroxine was studied in 17 euthyroid children between the ages of 3 and 9 years. Compared with adults, a more rapid turnover rate (13.9 per cent per day) was found. The amount of thyroxine degraded per day in micrograms per square meter of body surface was similar for children and adults although, per kilogram of body weight, children utilized more than did the adults.

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SERUM FACTORS IN LUPUS ERYTHEMATOSUS AND OTHER DISEASES REACTING WITH CELL NUCLEI AND NUCLEOPROTEIN EXTRACTS: ELECTROPHORETIC, ULTRACENTRIFUGAL AND CHROMATOGRAPHIC STUDIES

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In the past decade much interest has been focused on the factors in human serum which in many ways resemble antibodies against components of cell nuclei. The lupus erythematosus (LE) cell, discovered by Hargraves, Richmond and Morton in 1948 (1), is now known to result from the interaction of leukocyte nuclei and a factor(s) most frequently found in the serum of patients with lupus erythematosus. Miescher (2) employed the γ-globulin consumption test to demonstrate a reaction between factors in LE serum and isolated cell nuclei. Since this time, reactions between factors in LE serum and isolated cell nuclei (3, 4), intact nuclei in tissue sections (5–7), extracts containing nucleoprotein (8–10), nucleoprotein precipitates (11), preparations of desoxyribonucleic acid [DNA (4, 12–14)] and histone (15) have been demonstrated. The available information on the anti-DNA and other reactive factors in LE sera has been recently reviewed (16).

As more information concerning the serum-cell nucleus interactions has become available, the nature and the significance of the phenomena have appeared more complex. Anti-cell nucleus factors have been found in the sera of patients with diseases other than lupus erythematosus (7, 11), in sera which do not contain detectable LE cell factor. It appears, therefore, that not all serum-cell nucleus interactions are of the kind that bring about the changes necessary for the production of LE cells. In addition, recent studies (17, 18) indicate that the source of the tissue used in making nucleoprotein extracts is an additional variable affecting the detection of serum anti-nucleus factors. Certain sera were found to react with a *liver* nucleoprotein component which was heat-stable and was destroyed by desoxyribonuclease, whereas other sera reacted with a heat-labile component of nucleoprotein extracts made from *thymus* tissue. The present study was undertaken, therefore, to characterize in physicochemical terms several of the serum anti-nucleus factors, to determine whether the serum factors reacting with what appear to be different nucleoprotein materials might differ also in other properties, and to determine the relationship of the anti-nucleoprotein factors to the LE cell factor and other anti-nucleus factors.

Studies of the γ-globulins in man have emphasized that they are composed of two groups of proteins with ultracentrifugal sedimentation coefficients of 6.6S (molecular weight about 160,000) or of 18S (molecular weight about 1,000,000) (19). These groups contain a range of differing molecules (20). Anion-exchange cellulose chromatography is a useful means of separating the 18S γ-globulins from most of the 6.6S molecules and also provides a means for subdividing the 6.6S γ-globulin group (20). By chromatographic means, antibody activities associated with 18S protein molecules have been separated from smaller 6.6S antibody molecules (21–24).

In a preliminary report (24) on the anti-nucleus activities of LE sera, it was shown that certain anti-nucleus factors, presumably 18S γ-globulins, could be separated by anion-exchange cellulose chromatography from the LE cell factor which appeared in this and other (3, 25, 26) studies to be within the 6.6S γ-globulin group. The present report describes the electrophoretic, ultracentrifugal and chromatographic characterization of the factors in sera from patients with lupus erythematosus and other diseases which react with human leukocytes to form LE cells, with mouse liver cell nuclei as detected by fluorescein-labeled

TABLE I

Results of anti-cell nucleus and anti-nucleoprotein extract tests in sera from patients with systemic lupus erythematosus and other diseases

Diagnosis	No. of patients	No. of patients with positive LE cell test (human leukocyte)	No. of patients with positive anti-nucleoprotein tanned-cell hemagglutination test		No. of patients with positive fluorescein anti-liver nuclei (mouse) test
			Human liver nucleoprotein extract	Calf thymus nucleoprotein extract	
Systemic lupus erythematosus	27	25	15	23	26
Rheumatoid arthritis	30	2	6	3	6
Scleroderma	4	0	0	1	2
Other diseases*	53	0	0	0	6

* Includes idiopathic nephrotic syndrome, 17; dysgammaglobulinemias (principally macroglobulinemia), 13; convalescent mumps, 3.

antibody techniques, and with liver or thymus nucleoprotein extracts in tanned-cell hemagglutination tests.

METHODS

The tannic acid hemagglutination test was performed essentially as described by Boyden (27). Human type O Rh negative red cells or sheep red blood cells were used. The washed cells were "tanned" by mixing with a 1: 25,000 solution of tannic acid in pH 7.2 buffered saline for 30 minutes at room temperature. The tanned red cells, after washing, were coated by mixing with the nucleoprotein extracts for 30 minutes at room temperature. Nucleoprotein extracts were made from human liver obtained at autopsy and from calf thymus; the method for extraction of nucleoproteins with solutions of low ionic strength, described by Chargaff (28), was followed closely. All extracts were clarified by centrifugation in the Spinco model L preparative ultracentrifuge and stored frozen until used. The extracts were diluted from 1: 10 to 1: 400 with 0.15 M NaCl solution buffered at pH 7.2 before coating the tanned red cells; the concentration chosen was the highest that would not produce spontaneous agglutination of the red cells. For determination of anti-thyroglobulin titers, tanned red cells were coated with a 0.02 per cent solution of a thyroglobulin extract. The thyroglobulin was 86 per cent pure.

Quantitative total serum protein measurements, analytic paper electrophoresis and preparative electrophoretic procedures utilizing polyvinyl chloride particle blocks have been described (20). Ultracentrifugal fractionation was carried out in the manner of Robbins, Petermann, and Rall (29) utilizing a Spinco model L preparative centrifuge. Sera were diluted 1: 10 with 0.15 M NaCl solution and serum fractions were dialyzed thoroughly against the same solution prior to ultracentrifugation in the Spinco 40 rotor at 39,000 rpm for 150 minutes. At the end of this time successive samples were collected from the top to the bottom of the ultracentrifuge tube by using a sampling syringe and needle. Determinations were made of the anti-nucleoprotein extract activity, and the results were plotted in terms of the cumulative activity with increasing depth of the tube.

Anion-exchange cellulose chromatography was performed on columns of diethylaminoethyl (DEAE) cellulose (30), employing 2 g of adsorbent and 1 ml of serum. The starting and equilibrating potassium buffer was 0.02 M phosphate, pH 8.0. A 150 ml gradient elution system, providing a progressive rise in phosphate molarity to 0.25 M while maintaining pH 8, was used to elute the adsorbed proteins from the column (20). Effluent from the column was collected in about 45 fractions and the protein content estimated by measuring the optical density at 280 mμ in a Beckman spectrophotometer. The effluent was then combined into 10 or more pools which were tested for anti-nucleoprotein extract activity after dialysis against 0.15 M NaCl and without concentration.

For the fluorescent antibody technique, a $1 \times 1 \times 0.5$ cm piece of mouse liver was placed in a dry ice ethanol bath at $-70°$ C as quickly as possible after removal from the animal. When frozen, the tissue was transferred to a cryostat and sectioned at 5 μ at $-12°$ C. The sections were air-dried and then fixed in acetone for 10 minutes. Various dilutions of the patient's serum to be tested were put on the mouse liver sections and allowed to stand for 20 minutes at room temperature. The serum was then washed off with buffered saline, pH 7.4, with 3 changes of the saline at 5-minute intervals. Fluorescein-labeled anti-human globulin [1] was then placed on the section and allowed to react for 20 minutes at room temperature. The sections were again washed as before with buffered saline, mounted in 25 per cent glycerin in buffered saline and examined microscopically under ultraviolet light at a wave length of 390 to 440 mμ. The greatest dilution of the serum that produced fluorescence of the mouse liver cell nuclei was considered the titer of that serum.

[1] The fluorescein-conjugated horse anti-human globulin was obtained from the Progressive Laboratories, Baltimore, Md. This serum had been previously adsorbed with liver powder to remove nonspecific fluorescence.

RESULTS

Serological studies. Sera from 114 patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), scleroderma, idiopathic nephrotic syndrome and other diseases were examined. The results of LE cell tests, the titer of reactivity of the sera with nucleoprotein extracts made from human liver and calf thymus, and the titer of reactivity with mouse liver cell nuclei as determined by fluorescein-labeled anti-human globulin tests are listed in Table I. The LE cell test was positive in most cases of SLE and rarely positive in other diseases. Anti-human liver nucleoprotein extract activity was detected in 56 per cent of the patients with SLE and 20 per cent of a selected series of patients with RA. Anti-calf thymus nucleoprotein extract activity was present cent of the patients with SLE and in 20 per cent of RA sera and 25 per cent of the scleroderma sera. The fluorescein-labeled antibody to human γ-globulin revealed anti-cell nucleus activity in 96 per cent of the SLE sera, 20 per cent of the RA sera, 50 per cent of the scleroderma sera and 11 per cent of the other sera tested. The six positive sera in the last group included four patients with idiopathic nephrotic syndrome.

In view of this diversity of results when a variety of serological tests for anti-nuclei and anti-nucleoprotein extract activity was employed, a group of six sera was selected for further study (Table II).

The sera of the second group of three subjects (P.N., S.R. and M.F.) were selected because they reacted very strongly with the nucleoprotein extract made from calf thymus but not at all, or practically not at all, with the human liver nucleoprotein extract. All three sera also reacted with cell nuclei in the fluorescein antibody test, although the titer of serum S.R. seemed lower than the others. Serum M. F., from a patient with scleroderma, did not contain detectable LE cell factor.

The sera of the first group of three subjects jects (A.N., J.B. and H.L.) were chosen because they reacted primarily with the human liver nucleoprotein extract. In addition, the three sera reacted with cell nuclei in the fluorescein antibody test at high titer. Serum H. L. was also found to contain a moderately high titer of anti-thyroglobulin activity, and was the only one of the 15 LE sera tested that contained anti-thyroglobulin activity. A comparison of the results obtained with these six sera shows that it would not be possible to predict, on the basis of the results in one test system, what the behavior of the serum would be in the other four test systems. This suggests that multiple factors were involved in the anti-nucleus and anti-nucleoprotein extract activity detected in these six sera.

Electrophoretic studies. Sera from Subjects A.N., J.B. and P.N. were fractionated by zone (block) electrophoresis. The fractions of the A.N. and J.B. sera were tested for reactivity with

TABLE II

Serological characteristics of six sera with anti-nucleus and anti-nucleoprotein extract activity chosen for further study

Patient	Diagnosis	LE cell test (human leukocyte)	Anti-nucleoprotein extract; tanned cell hemagglutination		Anti-liver nuclei (mouse); fluorescein antibody test	Anti-thyroglobulin extract; tanned cell hemagglutination
			Human liver nucleoprotein extract	Calf thymus nucleoprotein extract		
Group I						
1. P.N.	SLE*	+	80†	60,000†	>128†	<10†
2. S.R.	SLE	+	<10	100,000	16	<10
3. M.F.	Scleroderma	0	<10	3,000,000	>128	<10
Group II						
4. A.N.	SLE	+	1,280	160	>200	10
5. J.B.	SLE	+	1,280	80	>128	<10
6. H.L.	SLE	+	1.280	<10	64	6,250

* Systemic lupus erythematosus.
† Reciprocal of the greatest serial dilution of serum which gave a positive test.

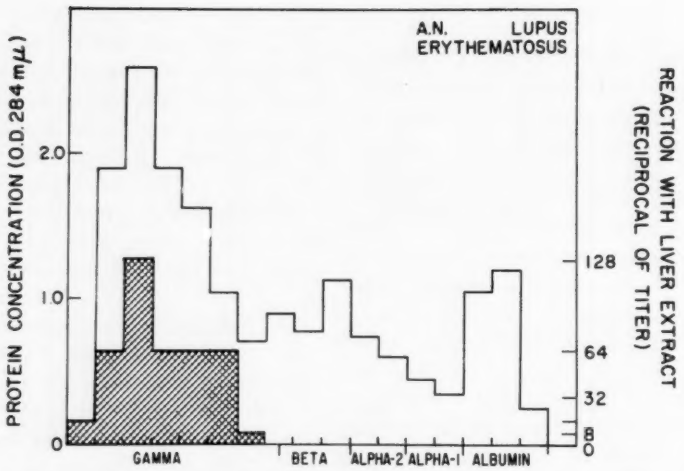

FIG. 1. DISTRIBUTION OF REACTIVITY WITH HUMAN LIVER NUCLEOPROTEIN EXTRACT AFTER ZONE (BLOCK) ELECTROPHORETIC FRACTIONATION OF A.N. SERUM. The shaded area represents the distribution of anti-nucleoprotein extract activity. The distribution of serum proteins was roughly determined by the location of the pigment bands, pink in the β region, blue in the α_2 region, and pale yellow in the albumin region, and was confirmed by analytical electrophoresis of the block sections.

human liver nucleoprotein extract and the fractions of serum P.N. were tested with calf thymus nucleoprotein extract. In each instance, serological activity was associated with the γ-globulin electrophoretic region as illustrated for serum A.N. in Figure 1. This serum contained an increased concentration of γ-globulins, 4.5 g per 100 ml.

Ultracentrifugal studies. Four sera were fractionated in a Spinco preparative ultracentrifuge. Ultracentrifugation was conducted in such a manner that most of the 18S γ-globulins, but little of the 6.6S proteins, would be concentrated at the bottom of the tube. The results are presented graphically in Figure 2.

In this figure, the *cumulative* percentage of serological reactivity is plotted against the distance from the top of the tube. The shaded area represents the behavior of the 6.6S γ-globulin anti-thyroglobulin activity in five sera of patients with chronic thyroiditis which were studied in the same way (23). The sedimentation properties of the anti-calf thymus extract activity in serum P.N. closely resembled those of the 6.6S anti-thyroglobulin activities. Very little of the activity had settled to the bottom of the centrifuge tube.

Anti-human liver nucleoprotein activity, on the other hand, sedimented more rapidly. A large proportion of this activity in sera A.N., J.B. and H.L., the sera of Group II, was found at the bottom of the centrifuge tube, in contrast to the anti-thyroglobulin activity which was also present in H.L. serum, and in contrast to the anti-thymus extract activity of serum P.N. This greater sedimentation of anti-liver nucleoprotein activity in whole serum could be due either to the association of this activity with a larger molecule or the distribution of this activity between both 6.6S and larger molecules. Data to be presented below suggest that the latter explanation is correct.

Chromatographic studies. Whole sera of γ-globulins obtained by preparative (block) electrophoresis from each serum were subjected to anion-exchange cellulose chromatography and the chromatographic fractions were tested for anti-nucleoprotein extract activity, anti-cell nucleus activity, and for capacity to form LE cells. Representative results are illustrated in Figures 3, 4 and 5. Consistent differences were observed between the sera of Group I, reacting primarily with thymus nucleoprotein extracts (Figures 3 and 5), and the sera of Group II, reacting with human liver nu-

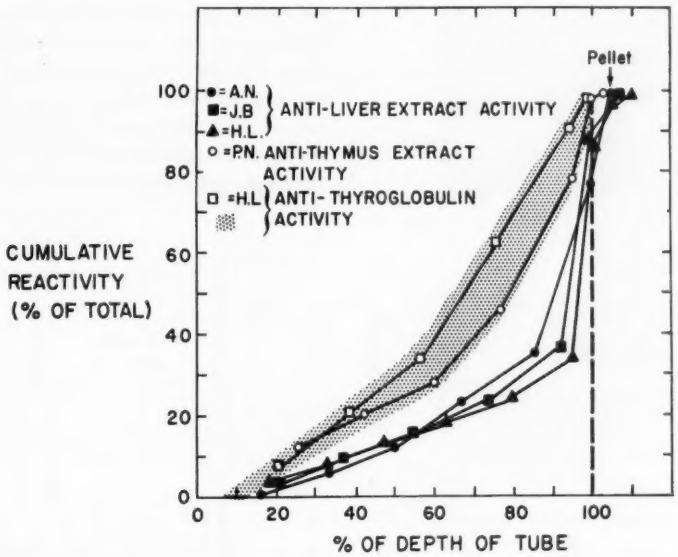

FIG. 2. SEDIMENTATION CHARACTERISTICS OF ANTI-NUCLEOPROTEIN EXTRACT ACTIVITIES IN WHOLE SERUM. Ultracentrifugation was conducted in a preparative centrifuge (39,000 rpm, 150 minutes, 0.15 M NaCl). After centrifugation, successive samples were removed from each tube starting at the top. The distribution of serological reactivity is expressed in terms of *cumulative per cent* of the total in each tube. The shaded area is the distribution of anti-thyroglobulin activities found in a concurrent study of five sera from patients with chronic thyroiditis (23).

cleoprotein extracts (Figures 4 and 5). In the Group I (anti-thymus) sera, almost all reactivity was found between 5 and 15 per cent of the effluent volume and all reactivity was found within 40 per cent of the effluent volume, while in the Group II (anti-liver nucleoprotein) sera the activity (except for LE cell factor) was found in both the 5 to 15 per cent and the 50 to 80 per cent effluent regions.

A comparison of the chromatographic elution patterns for whole serum and for γ-globulins prepared from whole serum (Figures 3 and 4) demonstrates that most of the γ-globulins are eluted in the region of 5 to 15 per cent of the effluent volume. Under the conditions of chromatography employed here, only γ-globulins with sedimentation coefficients of 6.6S are eluted in this region (20). In the Group I sera (P.N., S.R. and M.F.), anti-calf thymus nucleoprotein extract activity was found only in the initial portion of the chromatogram (Figure 3). In these sera, anti-cell nucleus activity was also found only in the 5 to 15 per cent effluent region. These findings, and the sedimentation of the anti-calf thymus extract activity when whole serum was ultracentrifuged, indicate that this activity and the anti-mouse liver cell nucleus activity in these sera are properties of γ-globulins with sedimentation coefficients of 6.6S.

In the Group II sera (A.N., J.B. and H.L.) the factors reacting with liver nucleoprotein extracts were eluted in the region of 5 to 15 per cent and 50 to 80 per cent of the effluent volume. As is seen in Figure 4, the distribution of activity is similar when whole serum or γ-globulin is chromatographed, an observation consistent with the results of electrophoretic fractionation showing the activity to be within the γ-globulin group. The finding of two chromatographic regions with activity indicates that in these sera the anti-nucleoprotein activity was associated with two different groups of γ-globulin molecules.

Ultracentrifugal studies of these two regions from the LE sera chromatograms were undertaken

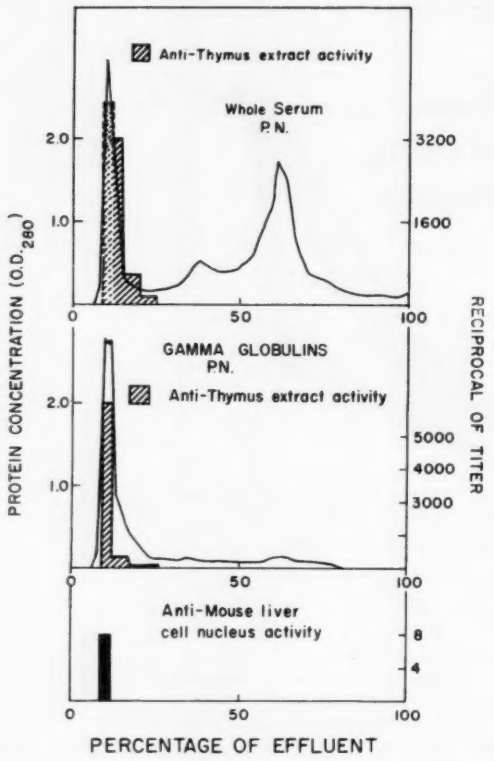

FIG. 3. CHROMATOGRAPHIC DISTRIBUTION OF SEROLOGICALLY DETERMINED ANTI-CALF THYMUS NUCLEOPROTEIN REACTIVITY IS SHOWN (TOP) IN WHOLE SERUM OF PATIENT P.N., AND (MIDDLE) IN THE γ-GLOBULIN OBTAINED BY PREPARATIVE ELECTROPHORESIS OF SERUM P.N. Also shown (bottom) is the anti-cell nucleus activity in the γ-globulin as determined by the fluorescent antibody technique. Chromatography was conducted by standard procedures on diethylaminoethyl-cellulose columns. Proteins were eluted by means of increasing phosphate concentration from 0.02 to 0.25 M, while pH was maintained at pH 8.0.

to determine whether the anti-nucleoprotein activities of these regions were associated with proteins of different sedimentation properties.

Previous studies (20) had shown that the γ-globulin eluted in the 50 to 80 per cent region of the chromatogram was composed primarily of 18S γ-globulins in contrast to the 5 to 15 per cent effluent region which contained only 6.6S γ-globulins. In Figure 6 the ultracentrifugal properties of the proteins contained in the 5 to 15 per cent and the 60 to 80 per cent region of the chromatogram of L.B. γ-globulins is shown. The proteins eluted first are seen to sediment less rapidly ($s^0_{20,w} \cong 18S$) than those eluted in the 60 to 80 per cent effluent region ($s^0_{20,w} \cong 18S$). Confirmation that the anti-nucleoprotein activity was similarly divided between faster and slower sedimenting γ-globulin components was obtained by

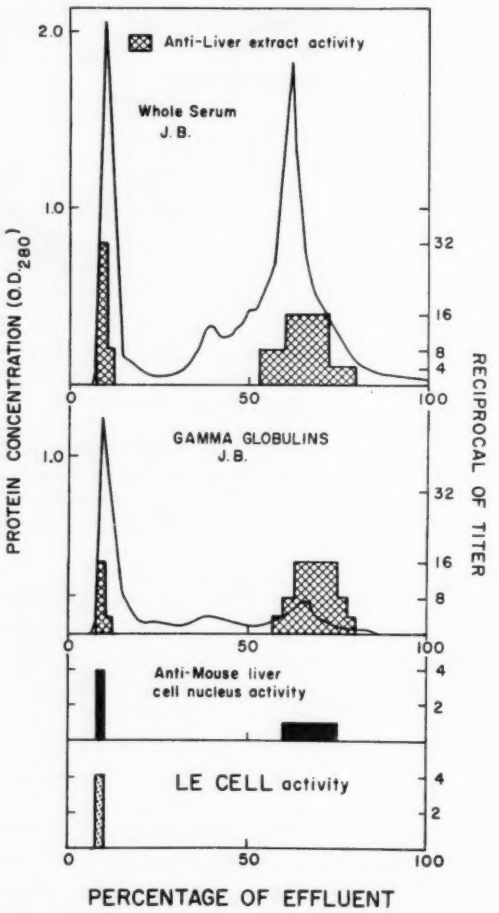

FIG. 4. CHROMATOGRAPHIC DISTRIBUTION OF SEROLOGICAL REACTIVITY WITH HUMAN LIVER NUCLEOPROTEIN EXTRACT IN WHOLE SERUM OF PATIENT J.B. AND IN γ-GLOBULINS PREPARED FROM SERUM J.B. Activity is found in two chromatogram regions. Below, the anti-nucleus activity (by the fluorescent antibody technique) is shown in the two chromatogram regions of the γ-globulins, and (bottom) lupus erythematosus (LE) cell activity is found in only one chromatogram region of the γ-globulins. Chromatography procedure was the same as that described for Figure 3.

preparative ultracentrifugal fractionation. In Figure 7 is illustrated the rapid sedimentation of the anti-human liver nucleoprotein activity in the 60 to 80 per cent effluent fraction which contrasts with the slower sedimentation behavior of the activity in the 5 to 15 per cent chromatogram effluent region. The agreement between the sedimentation behavior of the serological activities and the behavior of the proteins in these two regions supports the conclusions that the anti-liver nucleoprotein activity is associated with two groups of γ-globulin molecules, those with sedimentation coefficients of 6.6S and 18S, and that these two groups have been separated by anion-exchange cellulose chromatography.

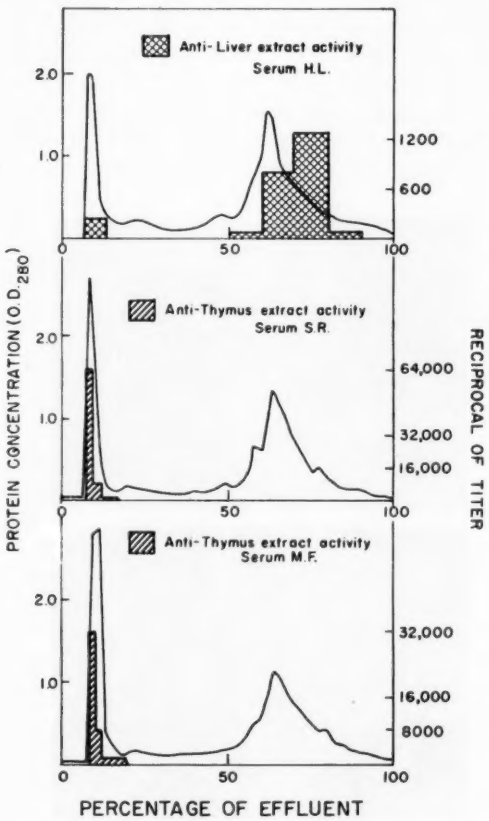

FIG. 5. CHROMATOGRAPHIC DISTRIBUTION OF SEROLOGICAL REACTIVITY WITH HUMAN LIVER EXTRACT IN WHOLE SERUM H.L. WITH ACTIVITY FOUND IN TWO CHROMATOGRAM REGIONS; BELOW IS SHOWN THE CHROMATOGRAPHIC DISTRIBUTION OF REACTIVITY WITH CALF THYMUS EXTRACT IN WHOLE SERA S.R. AND M.F., WITH REACTIVITY IN ONLY ONE REGION. Chromatography procedures were the same as described for Figure 3.

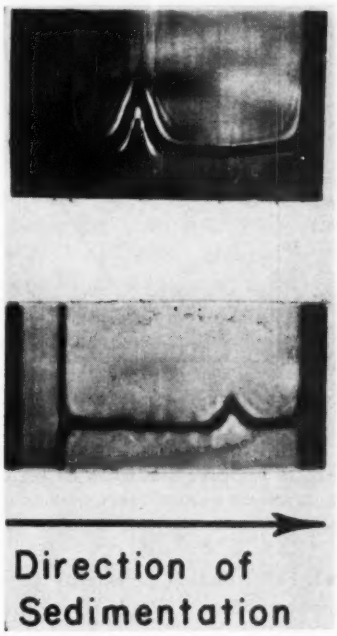

FIG. 6. ULTRACENTRIFUGAL ANALYSIS OF THE 5 TO 15 PER CENT REGION (UPPER) AND IN THE 60 TO 80 PER CENT EFFLUENT REGION (LOWER) FROM AN ANION-EXCHANGE CELLULOSE CHROMATOGRAM OF ELECTROPHORETICALLY PREPARED γ-GLOBULIN FROM L.B. SERUM. The photographs were obtained 26 and 24 minutes, respectively, after reaching rotor speed of 59,780. The direction of sedimentation is to the right. Samples had been dialyzed against 0.15 M NaCl prior to analysis. The sedimentation coefficient was approximately 6.6S for the component in the upper, and 18S in the lower photographs.

If the results of the preparative ultracentrifugal analysis of the two J.B. γ-globulin components illustrated in Figure 7 are compared with the results when J.B. whole serum was similarly analyzed (Figure 2), it is seen that the whole serum results fall between the findings for the individual components. This observation is consistent with the interpretation that the pattern of distribution of anti-nucleoprotein extract activity of this type (anti-human liver) after centrifugation of whole serum (Figure 2) is due to the presence of two components. The existence of two ultracentrifugally different components may be suspected when the whole serum results do not conform to

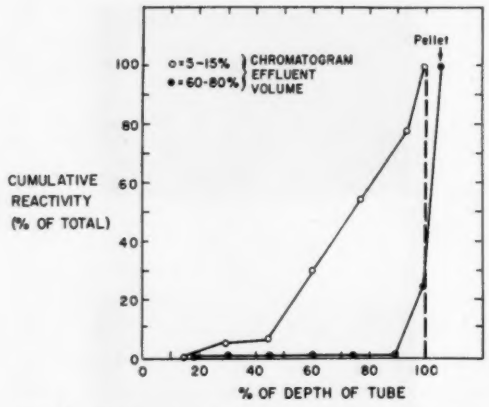

FIG. 7. DIFFERENCES IN THE SEDIMENTATION CHARACTERISTICS OF TWO ANTI-HUMAN LIVER EXTRACT COMPONENTS (5 TO 15 PER CENT AND 60 TO 80 PER CENT) OBTAINED BY CHROMATOGRAPHIC FRACTIONATION OF SERUM J.B. Ultracentrifugation was carried out as in Figure 2.

the expected distribution of activity for 6.6S or 18S components alone. Whole serum preparative ultracentrifugation, however, is a relatively crude technique, and small amounts of one type of γ-globulin activity would be difficult to detect in the presence of large amounts of the other type of γ-globulin activity. Anion-exchange cellulose chromatography provided a better means of separating and detecting the presence of both 6.6S and 18S γ-globulin activities.

Fluorescein-labeled anti-human globulin tests. The γ-globulins of the patient's sera which combined with mouse liver nuclei were detected by their reaction with fluorescein-labeled anti-human globulin. Examples of positive and negative results are illustrated in Figure 8. In the Group I sera (reacting with calf thymus nucleoprotein extract) the anti-cell nucleus factors were found only in the 5 to 15 per cent chromatogram effluent region (Figure 3), whereas in the Group II sera (reacting serologically with liver nucleoprotein extract) the positive fluorescent anti-globulin tests were obtained in both the 5 to 15 per cent and 50 to 80 per cent effluent regions (Figure 4). This shows that at least two physicochemically different γ-globulin components in these latter sera give positive fluorescein antiglobulin reactions and, on the basis of the observations noted above, it seems probable that the two components are 6.6S and 18S γ-globulins.

Some of the activities detected by the fluorescein anti-globulin test may be the same as those detected by the serological anti-nucleoprotein extract tests. Very likely several activities are involved, for inspection of the data in Table II reveals that the activities detected by these two types of tests were not increased equally in LE sera.

LE cell tests. The γ-globulin components responsible for LE cell formation were present only in the 5 to 15 per cent chromatogram effluent region [Figure 4 and Patient H.L. (*22*)]. As only 6.6S γ-globulins are found in this chromatogram region, the factor(s) responsible for LE cell formation must be 6.6S γ-globulins. The two sera fractionated for detailed study of the factors responsible for LE cell formation were chosen because they had both 6.6S and 18S γ-globulins which reacted with nuclear components by serological and histochemical tests (Group II sera). In view of the absence of LE cell factor in ma-

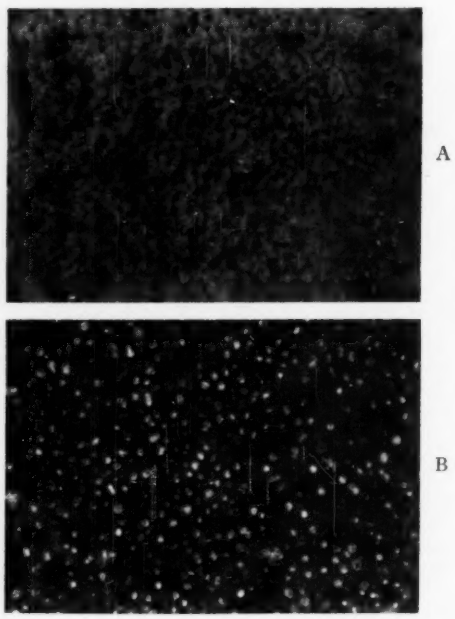

FIG. 8. MOUSE LIVER SECTIONS EXPOSED TO HUMAN SERUM, WASHED, EXPOSED TO FLUORESCEIN-LABELED ANTIBODY TO HUMAN γ-GLOBULIN, AND WASHED AGAIN. A. Lack of fluorescence of the cell nuclei after exposure to normal serum (cell nuclei appear as dark holes) when observed with an ultraviolet light source. B. Bright fluorescence of the nuclei after exposure to lupus erythematosus serum. 150× magnification.

terial from the 50 to 80 per cent effluent region which contained factors reacting with nucleoprotein extract and cell nuclei, it is clear that the LE cell factor has been separated chromatographically from some of the other anti-nucleus factors in these sera.

DISCUSSION

The discussion of the multiple serum factors reacting with tissue components in diseases such as lupus erythematosus is complicated by the lack of exact knowledge of the tissue substances with which the sera react. The crude nucleoprotein extracts employed could well contain cytoplasmic contaminants, and in fact, Deicher, Holman and Kunkel (31) have recently reported complement fixation reactions with liver cell mitochondrial preparations in the 19S globulins of one of eight SLE sera tested. Although in the present experiments no definitive studies were made of the nature of the active substances extracted from calf thymus because of their lability, the substances in human liver extracts with which the sera reacted were found to be very heat-stable and were destroyed by incubation with desoxyribonuclease (17); they were assumed to be nucleoproteins. In any case, whatever reactions with cytoplasmic constituents may have been present, all six of the sera studied here in detail reacted with components of cell nuclei as shown by the fluorescein-anti-globulin test. Each differed from the others, however, in some aspect of its reactions with cell nucleus components. The sera were considered in two groups. Three of the sera were in Group I, reacting with nucleoprotein extracts of calf thymus and showing activity only within the 6.6S γ-globulins. The three sera of Group II reacted primarily with nucleoprotein extracts from human liver and contained this activity in both 6.6S and 18S γ-globulins. This latter group of sera also contained two types of γ-globulin reacting with the mouse liver cell nucleus.

These observations could be explained either on the basis of a fundamental difference in immune response between the two groups of patients (i.e., those in Group I are limited to the manufacture of 6.6S antibodies, whereas those in Group II can also make 18S antibodies) or on the basis that the two patient groups were subjected to different kinds of immune challenge. Although we cannot yet distinguish between these alternative explanations, there is some evidence that these patients did not have a strict limitation of their capacity to manufacture 6.6S or 18S antibody. Serum M.F., which contained only 6.6S γ-globulins reacting with components of cell nuclei, also contained a high titer of rheumatoid factor which has been shown to be a γ-macroglobulin (32). On the other hand, Patient H.L., who was capable of producing 18S globulins which reacted with human liver nucleoprotein extracts, manufactures only 6.6S γ-globulins reacting with thyroglobulin. Antibodies to thyroglobulin extracts, however, need not be only 6.6S γ-globulins and, indeed, some patients produce largely 18S reactive globulins (23). These observations suggest that the different activities found in LE sera may be related to different tissue substances and that the tissue substances themselves might help to determine whether the antibodies to them are to be 6.6S or 18S γ-globulins.

The finding that the LE cell factor can be separated from some of the other anti-nucleus factors in serum is consistent with the concept of the existence of multiple anti-nucleus factors in the serum of patients with lupus erythematosus (4, 13, 15, 24). It is clear, therefore, that some serum-cell nucleus interactions are of a type which do not lead to the production of LE cells; the reasons for this are not evident. The data showing that other anti-nucleus factors are more widely distributed than is the LE cell factor is consistent with previous reports (7, 11). It will be necessary to test large numbers of sera from each of many disease categories, as well as a large number of normal sera, for the presence of anti-nucleus factors in order to determine their actual distribution.

Although the γ-globulins which react with nuclei in the manner required for the production of LE cells have so far been found only among the 6.6S γ-globulins (3, 25, 26), it is now apparent that 18S γ-globulin components may react with cell nucleus components in lupus erythematosus. Anti-cell nucleus reactivity can be added to the list of antibody-like activities already found in this group of macroglobulins: the isohemagglutinins (22, 33), rheumatoid factor (32) and the anti-thyroglobulin "antibodies" found in the serum of certain patients with thyroiditis (23). The

nomenclature of these macroglobulins is not uniform, and the proteins known as 18S or 19S γ-globulins or γ_1-macroglobulins in the American literature are classified as β_2 M-globulins in the immunoelectrophoretic studies of Burtin and coworkers (34). However, immunochemical studies of the macroglobulins which react with nucleoprotein extracts were not done.

It will be interesting to determine whether any correlations can be made between clinical findings and the serological differences described in the present study. So far as can be determined in a preliminary study (35), no obvious differences in clinical state can be correlated with the different patterns of anti-nucleus factors. The significance of the appearance of these antibody-like proteins in the serum of patients with lupus erythematosus and other diseases is not known. The production in rabbits of what may be somewhat similar auto-antibody-like anti-nucleus factors, after immunization with foreign cell nucleus materials (17, 36), at least suggests the possibility that bacterial or viral infection might result, in certain susceptible individuals, in the production of antibodies to nucleoproteins or other materials which cross react with similar components in human cell nuclei.

The ease with which DEAE cellulose chromatography allows recognition and separation of the 6.6S and 18S reactive factors should facilitate investigation of the mechanism of their production as well as the role, if any, played by these serum factors in the initiation or maintenance of disease processes.

SUMMARY

Multiple factors in sera from patients with lupus erythematosus (LE) and other diseases were found to react with components of cell nuclei. These anti-cell nucleus factors were detected by serological tests employing calf thymus and human liver nucleoprotein extracts and by fluorescent anti-globulin tests with mouse liver tissue and by LE cell tests.

All of the serum factors were in the γ-globulins separated by zone electrophoresis. The anti-nucleus factors were further characterized by anion-exchange chromatography and in some cases by ultracentrifugal studies.

The LE cell factor and the anti-thymus nucleoprotein activity were found only among 6.6S γ-globulins. In contrast, anti-human liver nucleoprotein extract activity was found largely with the 18S γ-globulins, although some activity was also found with 6.6S γ-globulins. The anti-cell nuclei activity detected by the fluorescent anti-globulin test was found with the 6.6S γ-globulins in LE sera reacting with calf thymus nucleoprotein extract and was found with 18S and 6.6S γ-globulins in LE sera reacting primarily with human liver nucleoprotein extract.

The qualitative and quantitative variety of serum factors in lupus erythematosus was notable, although the reasons for their appearance and their role in diseases remains obscure. Anion-exchange cellulose chromatography provided an excellent means for separating and identifying the reactive 6.6S and 18S γ-globulins.

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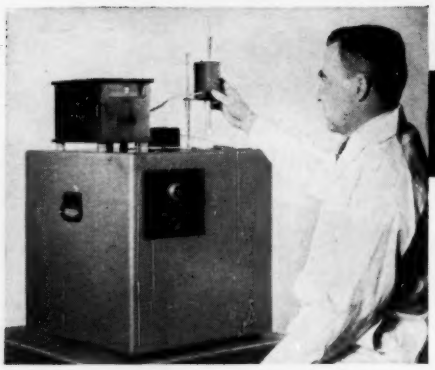

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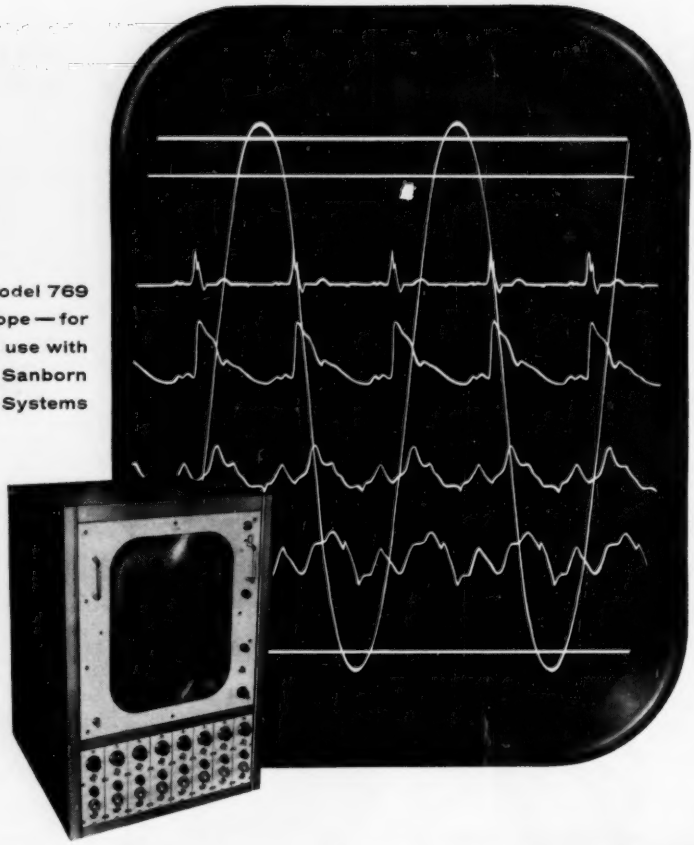

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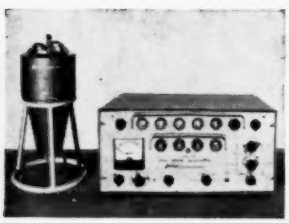

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